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NONVOLATILE ORGANICS IN DISINFECTED WASTEWATER EFFLUENTS: CHEMICAL CHARACTERIZATION AND MUTAGENICITY

R. L. Jolley, et al

Oak Ridge National Laboratory
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Principal objectives of this research program were to examine the effects of disinfection by chlorine, ozone, and ultraviolet light irradiation on nonvolatile organic constituents in secondary effluents relative to chemical effects and formation of mutagenic substances. In a comparative study of effluents from nine wastewater treatment plants, it was determined that disinfection with chlorine or ozone both destroys and produces nonvolatile organic constituents including mutagenic constituents. The chemical effects of disinfection by uv-irradiation were relatively slight although in one effluent, mutagenic constituents were eliminated by uv-irradiation. The investigation utilized the following methodology: concentration of effluents by lyophilization; high-pressure liquid chromatographic (HPLC) separation of nonvolatile organic constituents in effluent concentrates using uv-absorbance, cerate oxidation, and fluorescence detection; bacterial mutagenicity testing of concentrates and chromatographic fractions; identification and characterization of nonvolatile organic constituents in mutagenic HPLC fractions. No compounds known to be mutagens were identified in the mutagenic HPLC fractions separated from the undisinfected, chlorinated and ozonated effluent concentrates. The mutagenic activity of the nonvolatile organic constituents in one chlorinated effluent concentrate was not attributable to organic chloramines.

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FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. The complexity of the environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems for the prevention, treatment, and management of wastewater and solid and hazardous waste pollutant discharges from municipal and community supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research; a most vital communication link between the researcher and the user community.

Characterization of the non-volatile components in wastewater effluents, the largest segment of effluent composition - and determining their mutagenic potential enhances our knowledge of the makeup of our waterways and reflects on the efficacy of our wastewater treatment plants for removal of specific organics. This report provides a better insight on the composition of wastewater effluents; both disinfected and undisinfected and their mutagenic activities.

Francis T. Mayo
Director
Municipal Environmental Research
Laboratory

ABSTRACT

Principal objectives of this research program were to examine the effects of disinfection by chlorine, ozone, and ultraviolet light (uv) irradiation on nonvolatile organic constituents relative to chemical effects and formation of mutagenic substances.

In a comparative study of highly concentrated samples of effluents from nine wastewater treatment plants, it was determined that disinfection with chlorine or ozone both destroys and produces nonvolatile organic constituents. The chemical effects of disinfection by uv irradiation were relatively slight although in one effluent, mutagenic constituents were eliminated by uv irradiation.

The nine wastewater treatment plants were selected using the following criteria: disinfection method, nature of wastewater source, type of wastewater treatment, standards for quality of treatment, and geographical location. The treatment plants varied from pilot plant and small plants [0.05 m^3/s (1 Mgd)] treating principally domestic wastes to large plants [4.4 m^3/s (100 Mgd)] treating principally industrial waste. Four plants used only chlorine for disinfection, four plants used ozone for disinfection, and one plant used uv irradiation for disinfection. Eight treatment plants used conventional secondary or more advanced wastewater treatment and one plant used primary treatment.

The investigation used the following methodology: grab sample collection of 40-L samples of undisinfected and disinfected effluents; concentration of the effluents by lyophilization; high-pressure liquid chromatographic (HPLC) separation of nonvolatile organic constituents in effluent concentrates using uv absorbance, cerate oxidation, and fluorescence detectors; bacterial mutagenicity testing of concentrates and chromatographic fractions; and identification and characterization of nonvolatile organic constituents in mutagenic HPLC fractions.

Interplant comparison revealed considerable variability in the presence of mutagenic nonvolatile organic constituents in the undisinfected effluent concentrates as well as much variability in the destruction of the mutagenic constituents and the formation of other mutagenic constituents as a result of disinfection. Moreover, the effects varied on samples collected at the same wastewater treatment plant at different periods.

Although 104 compounds were identified, no compounds known to be mutagens were identified in the mutagenic HPLC fractions separated from the undisinfected, chlorinated, and ozonated effluent concentrates. The mutagenic activity of the nonvolatile organic constituents in one chlorinated effluent concentrate was not attributable to organic chloramines.

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Most of the mutagens detected in effluent concentrates are direct acting and do not require metabolic activation. Both base pair substitution mutagens and frame-shift mutagens occurred in the wastewater concentrates, but the former were more frequent. For many of the compounds in effluents, strain TA-1535 was more sensitive than strain TA-100 in detecting base pair substitution mutagens.

This report was submitted in fulfillment of Contract No. EPA-IAG-D7-01027 by Oak Ridge National Laboratory under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period January 1977 to March 1981, and work was completed as of March 31, 1981.

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SECTION 1

INTRODUCTION

The severity of waterborne diseases and disease epidemics has been greatly reduced because of disinfection treatment of wastewater and potable waters. During the last half century the principal disinfectant has been chlorine. It was only recently established that chloroorganics are formed when chlorine is used to disinfect wastewater effluents and potable waters.[1-6] These findings are attributable to the development of improved and more sensitive analytical methods in the last decade.

Because of the possible harmful effects of exposure to chlorinated organics,[7] there is considerable interest in the use of alternatives to chlorine. Currently, the principal alternative is considered to be ozone.[8,9] The chemistry of ozone reactions with specific organic chemicals has been studied extensively. But the effects of ozonation on wastewater and wastewater treatment plant effluents have been characterized principally in general terms such as oxidation of organic carbon, biochemical oxygen demand (BOD), and color.[9] Relatively little is known about the formation (or possible formation) of ozonation and ozonolysis products during disinfection with ozone, although the first products of ozone oxidation of organic molecules are presumed to be oxygenated materials that are water soluble and not highly volatile.[9] Additional information is needed before ozone can be evaluated as an alternative to chlorine. Another alternate disinfection method is irradiation with ultraviolet (uv) light. For this method to become a viable alternative, its effects on organic constituents in wastewater must be determined and compared with the effects of chlorine and ozone.

Evaluation of the toxicological effects of byproducts of chlorine, ozone, or uv irradiation in such complex matrices as effluents from wastewater treatment plants is a very arduous and complex problem.[10] Within the last decade rapid progress has been made in the analysis of volatile organic constituents in water and wastewater.[2,3,5] However, the determination and characterization of nonvolatile organics have not advanced as rapidly, even though the greatest portion of soluble organics in wastewater and natural water consists of compounds in this category.[11,12] This lack of analytical progress is probably due to the inherently greater complexity of the "nonvolatile" molecular constituents.[12] Nevertheless, an understanding of the possible environmental and health-related effects of nonvolatile organics in wastewater based on definitive data is essential.

Several Oak Ridge National Laboratory (ORNL) research and development programs have dealt with similar complex problems in the biological, biomedical, and environmental sciences. For example, in one such program, high-resolution liquid chromatographic systems were developed to analyze the molecular constituents in complex mixtures and solutions such as physiologic fluids. In the 3-year program, "Automated Analysis of Individual Refractory Organics in Polluted Water by High-Resolution Ion Exchange Chromatography," which was supported by the U.S. Environmental Protection Agency, the high-resolution analyzer was used and a multicomponent identification procedure was developed to characterize and identify many organic constituents in chlorinated and unchlorinated effluents of domestic wastewater treatment plants.[6] The techniques and methods used in these studies were particularly designed to examine those compounds having relatively low volatility.

Oak Ridge National Laboratory pioneered in the examination of the toxic properties of irradiation.[13] Some of the genetic test methods developed during these studies may be applied to the assessment of potential environmental damage from pollutants.[14,17] However, probably the most widely used and accepted assay for mutagenic potential of environmental pollutants is the bacterial mutagenesis system developed by Dr. Bruce Ames.[18,20] This *Salmonella* test system can yield reproducible data in analysis of complex mixtures.[10]

The objective of this research program was to examine the effects of disinfection of secondary effluents with chlorine, ozone, or uv-light irradiation on nonvolatile organic constituents. The program was divided into four major tasks:

1. Investigation of the effects of ozonation, chlorination, and uv-light irradiation on nonvolatile organics in effluents of a typical secondary wastewater treatment plant. The disinfection was done in the laboratory under carefully controlled conditions to achieve a fecal coliform level of 200 per 100 mL effluent. Conclusions derived from this study should thus be useful in assessing actual risk associated with each of the disinfection processes. High-resolution liquid chromatography was used to separate organic constituents from concentrates of samples both before and after the disinfection treatment. Chromatographic profiles were examined for differences due to disinfection.

2. Analysis of samples from actual operating wastewater treatment plants. Both analytical- and preparative-scale liquid chromatographic separations were made on samples before and after disinfection. The following sites were selected for collection of field samples based on disinfection method, nature of wastewater treated, and type and quality of treatment:

   a. Disinfection with ozone.

      Upper Thompson Sanitation District, Estes Park, Colorado.

      Taft Center, U.S. Environmental Protection Agency Pilot Wastewater Treatment Plant, Cincinnati, Ohio.

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Meander Creek Wastewater Treatment Plant, Mineral Ridge, Ohio.

Marlborough Easterly Advanced Waste Treatment Plant, Marlborough, Massachusetts.

b. Disinfection with uv light.

Northwest Bergen County Wastewater Treatment Plant, Waldwick, New Jersey.

c. Disinfection with chlorine.

Marlborough Easterly Advanced Waste Treatment Plant, Marlborough, Massachusetts.

Oak Ridge East and West wastewater treatment plants, Oak Ridge, Tennessee.

Lyons Bend Fourth Creek Wastewater Treatment Plant, Knoxville, Tennessee.

Moccasin Bend Wastewater Treatment Plant, Chattanooga, Tennessee.

3. Characterization and identification of constituents separated from effluents in the preceding tasks using a multicomponent analytical procedure, which includes gas chromatography and mass spectrometry.

4. Screening for mutagenic activity of concentrated effluent samples and selected components separated by liquid chromatography from the effluent samples. In the later phases of the research program, mutagenic activity was used as the criterion for characterization efforts on separated chromatographic components.

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SECTION 2

CONCLUSIONS

The occurrence of materials that are mutagenic to enteric bacteria in concentrates of wastewater effluents is widespread.

Disinfection of secondary effluents by chlorination was found to destroy some nonvolatile organic constituents and to produce others in the majority of samples examined. The mutagenicity of certain fractions was similarly affected. Chlorination of primary effluents produces far more mutagenic constituents than does chlorination of secondary effluents. Of the six treatment plants using chlorination, effluent concentrates from three plants showed increased mutagenicity and concentrates from two plants showed reduced mutagenicity after chlorination.

Disinfection of secondary effluents by ozonation both destroys and produces some nonvolatile organic constituents including those showing mutagenic activity in the majority of effluents examined. In this study, one primary effluent that produced increased mutagenic activity upon chlorination did not display such activity upon ozonation.

Disinfection of secondary effluents by uv irradiation has a definite but relatively slight effect on the nonvolatile organic constituents. Several mutagenic constituents in one undisinfected effluent were destroyed by uv irradiation. Disinfection of one primary effluent that produced increased mutagenic activity upon chlorination did not display such activity upon uv irradiation.

Samples collected from the same plant on different dates showed varying chemical composition and mutagenic components. Similarly varying characteristics were noted for effluents of different plants treated with the same disinfectant.

No compounds that had been previously reported as mutagenic were identified in the separated mutagenic HPLC fractions. The mutagenic activity of the nonvolatile organic constituents in one mutagenic chlorinated effluent concentrate was not attributable to organic chloramines. Most of the mutagens detected in effluent concentrates were direct acting and did not require metabolic activation. Both base pair substitution mutagens and frame-shift mutagens occurred in wastewater concentrates, but the former were more frequent. For many of the materials in the concentrates, strain TA-1535 was more sensitive than strain TA-100 in detecting base pair substitution mutagens.

Some mutagenic constituents were not separated by high pressure liquid chromatography (HPLC) with strongly basic anion exchange resin either because of irreversible adsorption on the resin or decomposition on the column. Conversely, chromatography of some effluent concentrates that tested negative in mutagenic activity tests resulted in separation of mutagenic HPLC eluate fractions. This phenomenon may be attributable to removal of the mutagenic constituent(s) from toxic components with a consequently more accurate bacterial mutagenicity test.

Fluorescence was evaluated as a detection method for HPLC chromatography of nonvolatile organic constituents in wastewater effluents. It was found to greatly enhance detection of chromatographed constituents.

SECTION 3

RECOMMENDATIONS

This study has established that some mutagenic nonvolatile organic constituents are produced by chlorination and ozonation of the majority of effluent concentrates studied. The scope and magnitude of this production should be established more clearly to assess its environmental and possible health significance.

Further chemical characterization studies are necessary to establish the identities of the mutagenic constituents present in the undisinfected effluent concentrates and those produced by chlorination or ozonation. Development of analytical methodology is needed to permit characterization and identification of the many phosphate-containing organic constituents and to better characterize the mutagenic constituents. More rapid and economic analytical and mutagenic bioassay techniques need to be developed in order to ensure adequate and economical investigation of disinfection effects on nonvolatile organic constituents in wastewater effluents.

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SECTION 4

SOURCES OF EFFLUENT SAMPLES

Nine wastewater treatment plants listed in Table 1 were selected as sites for collection of effluent samples. Consistent high standards for operation of the plant and quality of effluent were prime considerations in their selection. Six plants were operated under the auspices of U.S. Environmental Protection Agency programs that provided considerable information regarding treatment parameters and quality of the effluent. Details of each plant are given in the Appendix.

Principal criteria for this selection were the following:

1. Location. The treatment plants selected were within reasonable geographic distance of ORNL such that the effluent samples could be transported to ORNL within one to two days after collection.

2. Type of wastewater treatment. To check out procedures and determine experimental capabilities of the chemical characterization methods and bioassay sensitivities, a primary treatment plant with a well-characterized effluent was initially used. However, because secondary treatment has been mandated for most municipal wastewater treatment plants, plants with secondary or more advanced treatments, e.g., nitrification and filtration, were selected.

3. Disinfection method. A major objective of this program was to evaluate and compare the chemical effects of disinfection on nonvolatile organic constituents. Three modes of disinfection were investigated: chlorination, ozonation, and ultraviolet irradiation. Thus, the plants selected used one or two of these methods for disinfection of the final effluent.

4. Nature of wastewater source. In an attempt to make conclusions from this research as widely applicable as possible, an effort was made to include wastewater plants that treated principally domestic (residential) waste from small and large communities, mixed domestic and small business waste, and mixed domestic and large industry waste.

TABLE 1. SOURCES OF EFFLUENT SAMPLES

Designation	Name of treatment plant	Location	Type of treatment	Disinfection method	Type of wastewater
ORPE	Oak Ridge West Wastewater Treatment Plant	Oak Ridge, TN	Primary	Chlorine Ozone[a] uv[a]	Primarily residential
ORSE	Oak Ridge East Wastewater Treatment Plant	Oak Ridge, TN	Secondary[b]	Chlorine Ozone[a] uv[a]	Primarily residential
LBSE	Lyons Bend Fourth Creek Wastewater Treatment Plant	Knoxville, TN	Secondary[b]	Chlorine	Primarily residential
MBSE	Moccasin Bend Wastewater Treatment Plant	Chattanooga, TN	Secondary[b]	Chlorine	Residential and industrial
MMSE	Marlborough Easterly Advanced Waste Treatment Plant	Marlborough, MA	Secondary[b]	Ozone Chlorine	Primarily residential
EPSE	Upper Thompson Sanitation District	Estes Park, CO	Secondary[b]	Ozone	Residential, relatively clean
CSE	Taft Center, USEPA Pilot Wastewater Treatment Plant	Cincinnati, OH	Secondary[b]	Ozone	Residential and industrial
MCSE	Meander Creek Wastewater Treatment Plant	Mineral Ridge, OH	Secondary[b]	Ozone	Primarily residential
NBCSE	Northwest Bergen County Wastewater Treatment Plant	Waldwick, NJ	Secondary[b]	Chlorine uv	Primarily residential

[a] Conducted in laboratory tests.
[b] Activated sludge.

SECTION 5

METHODS AND METHODOLOGY DEVELOPMENT

The general methodology used for this research program is summarized in Table 2. A detailed description of the methods used and developed during this research program for the collection, treatment, and characterization of the wastewater plant effluent samples follows.

SAMPLE COLLECTION AND TRANSPORTATION

Effluent grab samples were collected by ORNL or, where practicable, by local personnel at the wastewater treatment plants. Sampling was scheduled to obtain effluent samples with the highest concentration of organics. To ensure that the disinfected effluent sample was representative, it was sampled after the control by a time period equivalent to the calculated residence time in the disinfection treatment. The samples were assigned an alphanumeric identification at ORNL and the sample information (e.g., sample type, date, time, flow characteristics, type of treatment) was recorded in a permanent log book. Effluent data provided by the treatment plant operators for all samples collected during this research program are given in the appendix.

Because of the large volume of the field samples (40 L per sample), and because several of the field sampling sites are geographically remote from ORNL, a field sampling protocol was developed to ensure the preservation of the samples in a refrigerated state during shipment. Six insulated, plywood shipping containers were constructed, each to contain two 7.4-L polyethylene containers.

Samples collected within close proximity of ORNL were delivered by car or air transportation without refrigeration. Otherwise, the samples were frozen with dry ice and shipped by air transportation to ORNL, usually arriving within 30 hr and still frozen. This latter method was used for field sampling at Estes Park, Colorado; Mineral Ridge, Ohio; Waldwick, New Jersey; and Marlborough, Massachusetts.

SAMPLE PROCESSING AND ANALYTICAL PROCEDURES

The preparation of samples for analysis, the separation of constituents, and the subsequent application of analytical methods to

TABLE 2. SUMMARY OF METHODS USED IN CHEMICAL CHARACTERIZATION AND MUTAGENICITY STUDIES

Operation	Method
Sample collection	Grab
Sample transportation	Air freight (-40°C) or car transportation (ambient temperature)
Concentration	Lyophilization
Separation	High-pressure liquid chromatography
Analytical	Mutagenicity Ultraviolet spectrophotometry Gas chromatography Gas chromatography/mass spectrometry

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separated fractions involved an integrated and complex series of manipulations and investigative techniques. The basic analytical steps used to examine the effluent samples were (1) concentration, (2) separation of constituents, and (3) characterization and identification of constituents. Generally concentration of effluents was required in order to obtain sufficient quantity of the separated constituents for subsequent testing. High-pressure liquid chromatography (HPLC) was used for separation of constituents in the concentrated effluents; while gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) were used for characterization and identification of the constituents.

Concentration of Nonvolatile Constituents

Depending on the uv-absorption properties of an individual compound, the lower limit of detection for the HPLC used in this program is in the microgram range. Because the HPLCs are limited to ≤ 5 mL per sample and the concentrations of specific contaminants in effluent samples may be ≤ 10 μg/L, it was necessary to concentrate wastewater effluents by factors of 1000 to 3000 prior to analysis. Initially in this program vacuum distillation was used as the concentration method. However, in an effort to achieve greater efficiency, two other alternative methods, adsorption on resin and lyophilization (freeze drying), were evaluated.[21,22] It was determined that lyophilization can be used in place of vacuum distillation to save both time and effort. Consequently, lyophilization was the principal concentration method used in this study. But in one test adsorption on macroreticular resins was found to be effective for concentrating mutagenic substances and, consequently, should be more thoroughly evaluted (see p. 67).

Vacuum Distillation--

The sequence of low-temperature distillation followed by two freeze-drying steps, as shown in Figure 1, was used in previous studies as the most convenient and suitable concentration method.[6] Samples were filtered through Whatman No. 2 filter paper to remove suspended matter; only negligible uv-absorbing material was lost in this separation. The volume of the filtrate was then reduced in the vacuum still at <35°C and ≤ 10 Pa (≤ 75 μm Hg) to about 150 mL of liquid plus some separated solids. The concentrated liquid, the solids, and a rinse solution were freeze dried. After freeze drying, the solids were acidified with acetic acid to destroy carbonate salts and centrifuged. The supernatant liquid was again transferred to the freeze dryer [<2 Pa (<15 μm Hg)], where the final reduction in volume was made. Water and acetate buffer were added to attain the desired liquid volume (20 to 40 mL) and to adjust the pH to 4.4. Finally, the resulting sample was well mixed, and the solids were separated by centrifugation. The supernatant liquid (effluent concentrate) was used for liquid chromatography and for mutagenic activity tests.

Lyophilization--

Because a commercial-size lyophilizer was available at ORNL, a comparison was made of concentration by vacuum distillation and by

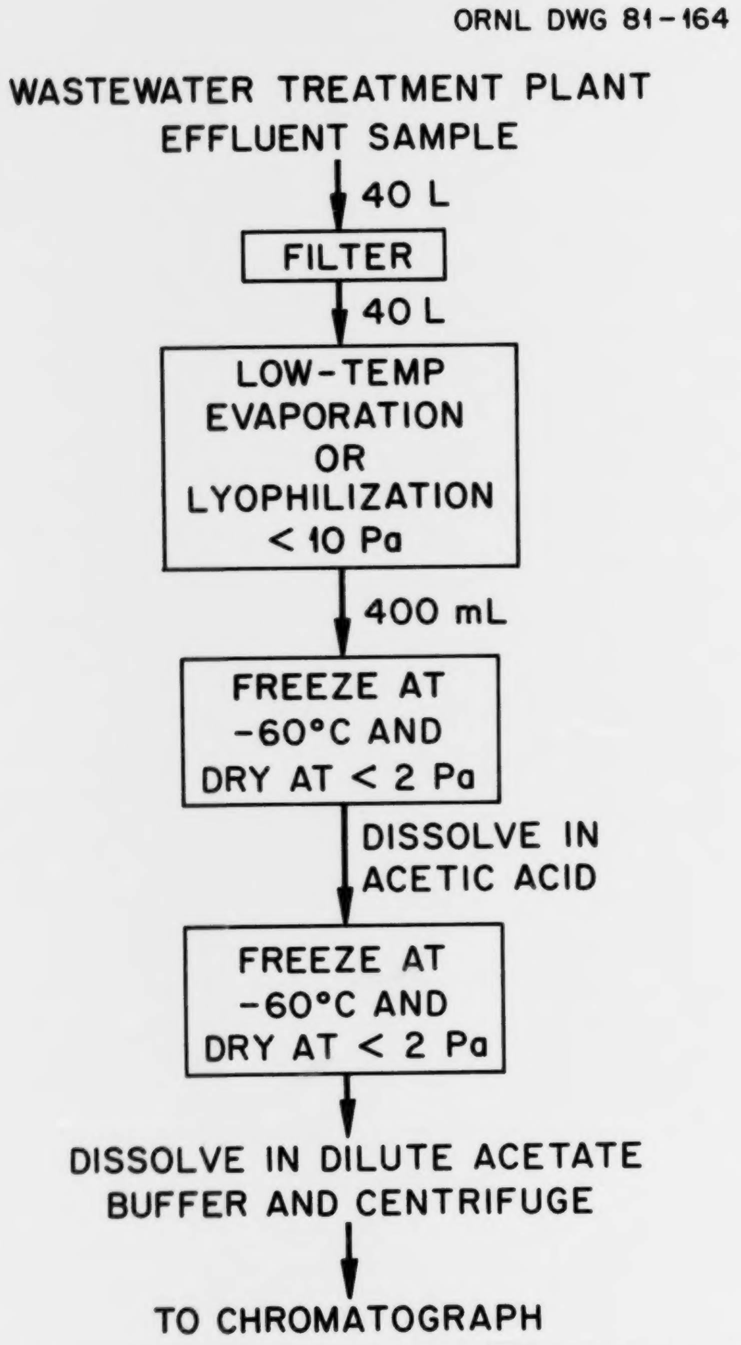
ORNL DWG 81-164
WASTEWATER TREATMENT PLANT EFFLUENT SAMPLE
40 L
FILTER
40 L
LOW-TEMP EVAPORATION OR LYOPHILIZATION < 10 Pa
400 mL
FREEZE AT -60°C AND DRY AT < 2 Pa
DISSOLVE IN ACETIC ACID
FREEZE AT -60°C AND DRY AT < 2 Pa
DISSOLVE IN DILUTE ACETATE BUFFER AND CENTRIFUGE
TO CHROMATOGRAPH

Figure 1. Procedure for concentrating effluent samples.

lyophilization. One 20-L aliquot of the primary effluent from the Oak Ridge East Wastewater Treatment Plant (collected 6/19/77, Table 43, Appendix) was concentrated by vacuum distillation and a second 20-L aliquot was concentrated by lyophilization. Superimposed chromatograms of each concentrate are shown in Figure 2. Unfortunately, comparison is complicated by differences in initial chromatographic eluent flow rates which appears to have "stretched out" the chromatographic constituents of the lyophilized concentrate in the first third of the chromatogram. Nevertheless, most of the major peaks are common to both samples. Operation of the lyophilizer requires less labor and attention than vacuum distillation. Moreover, there is less possibility of artifact formation in this process than in vacuum distillation as the latter requires heating the sample to 35°C while the lyophilization process maintains the sample at 0°C. It was thus decided that future samples be concentrated by lyophilization. The sequence of sample processing is exactly the same as those for vacuum distillation as shown in Figure 1. The large-scale lyophilizer used was capable of handling 100 L at a time.

Separation of Nonvolatile Constituents

High-pressure liquid chromatography was selected as the principal method for the isolation and separation of the nonvolatile constituents from the wastewater effluent concentrate.

HPLC Methodology--

Both preparative-scale and analytical-scale chromatographs (Figure 3) were used to analyze effluent concentrate samples. Two types of detectors were initially used: a uv detector for detection of uv absorbing constituents, and a cerate fluorescence (cerate oxidimetry) detector to detect cerate-oxidizable compounds (e.g., carbohydrates, organic acids, phenols). In the cerate fluorescence detector, cerium (IV) is reduced to fluorescent cerium (III) by the oxidizable compounds. Later a fluorometer was used in series with the uv detector to detect compounds that possessed the property of natural fluorescence.

The chromatographs consisted primarily of a heated, high-pressure ion exchange column; a sample injection valve; a two-wavelength, dual-beam uv photometer; a cerate oxidative monitor, and a strip-chart recorder. Physical characteristics and operating parameters for the HPLC systems are given in Table 3. The ion exchange column for each system was a 50-cm length of type 316 seamless stainless steel tubing (0.45 to 1.0 cm ID), usually packed with strongly basic anion exchange resin. A 0.05- to 5.0-mL sample (the volume depended on the inside diameter of the ion exchange column and the nature of the sample) was applied to the column by a six-port injection valve mounted as near to the top of the column as possible to minimize peak broadening.[6] Generally, 0.05-mL samples were used in analytical-scale chromatography and 5.0-mL samples were used in preparative-scale chromatography.

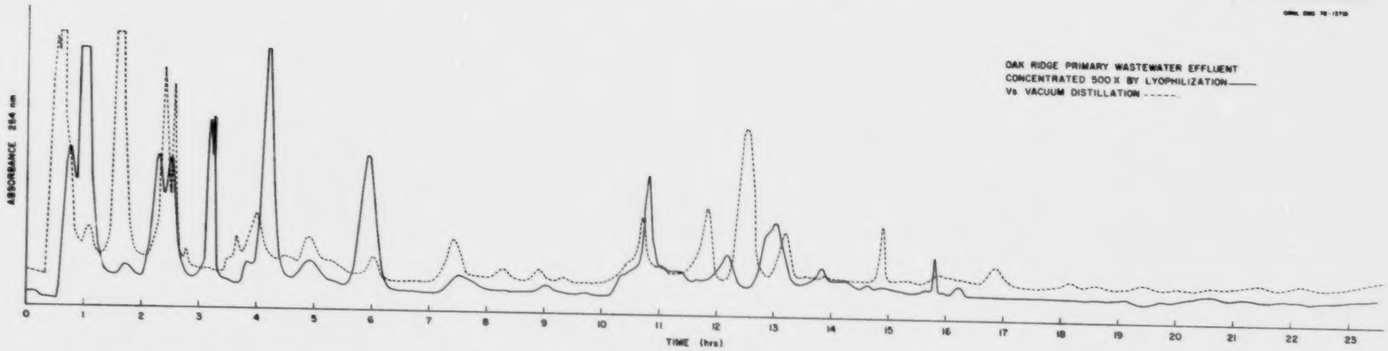

Figure 2. Comparison of chromatograms of concentrates of a primary effluent prepared by lyophilization and vacuum concentration. Both samples were concentrated 500 x.

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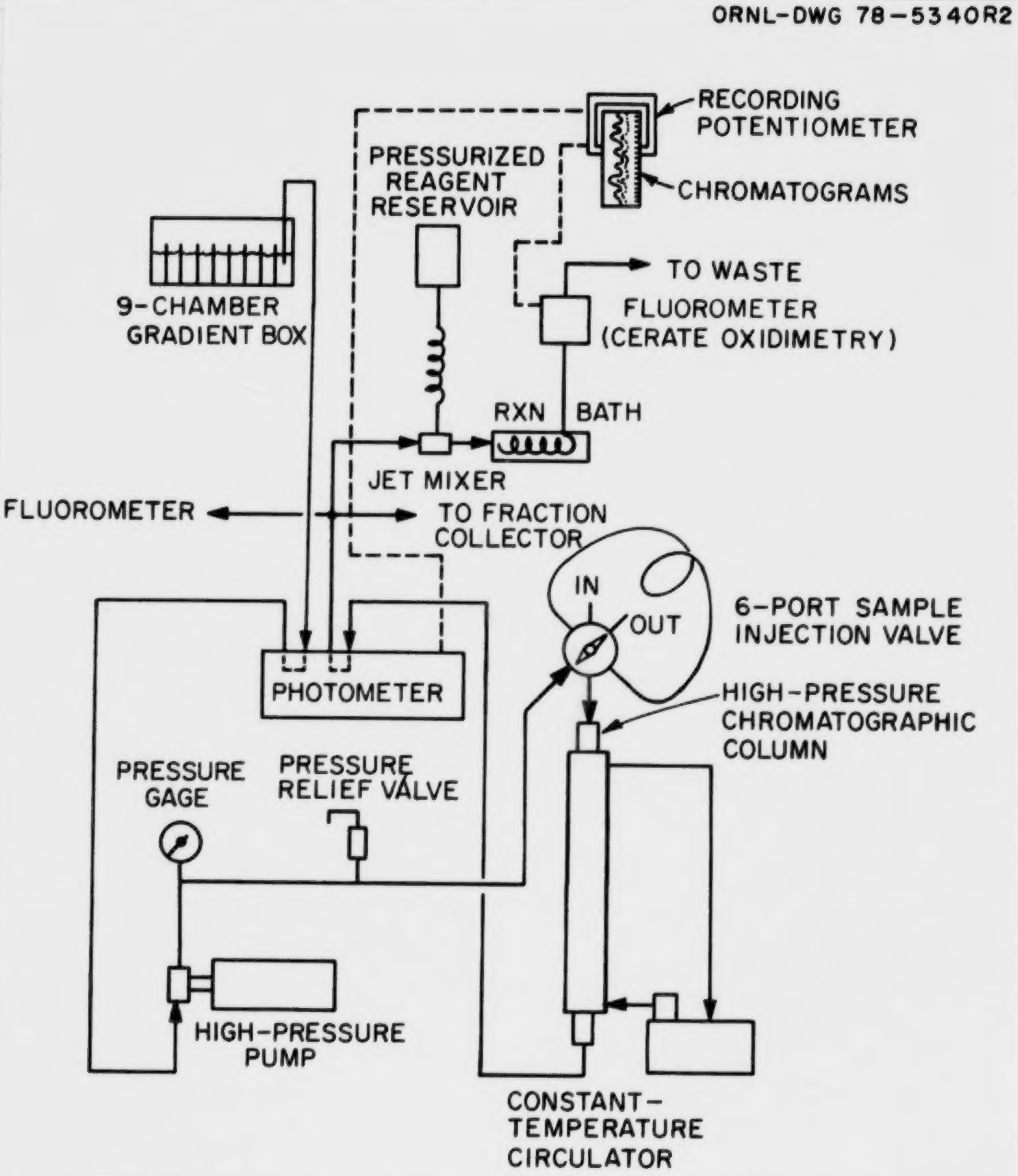
ORNL-DWG 78-5340R2
RECORDING POTENTIOMETER
CHROMATOGRAMS
PRESSURIZED REAGENT RESERVOIR
9-CHAMBER GRADIENT BOX
TO WASTE
FLUOROMETER (CERATE OXIDIMETRY)
RXN BATH
JET MIXER
FLUOROMETER
TO FRACTION COLLECTOR
IN
OUT
6-PORT SAMPLE INJECTION VALVE
PHOTOMETER
HIGH-PRESSURE CHROMATOGRAPHIC COLUMN
PRESSURE GAGE
PRESSURE RELIEF VALVE
HIGH-PRESSURE PUMP
CONSTANT-TEMPERATURE CIRCULATOR

Figure 3. Schematic of high-pressure liquid chromatographic systems. Three LC detectors are shown: photometer for uv-absorbing constituents; fluorometer for natural (native) fluorescent constituents; and cerate oxidimetry fluorometer [with associated reaction (RXN) bath] for cerate oxidizable constituents.

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TABLE 3. PHYSICAL CHARACTERISTICS AND OPERATING PARAMETERS FOR THE ANALYTICAL AND PREPARATIVE ANION EXCHANGE COLUMNS

Parameters	Columns[a]	
	Analytical	Preparative
Column dimensions (cm)	0.45 ID x 50	1.1 ID x 50
Resin	Bio-Rad A-27	Bio-Rad A-27
Resin particle size (μm)	12-15	12-15
Sample size (mL)	0.05	5
Run time (hr)	20	24
Temperature program	Ambient, increasing to 60°C after 1.5 hr	Ambient, increasing to 60°C after 0.5 hr
Operating pressure (psig)	500	500-1000
Operating pressure (Pa)	3.4×10^6	$3.4\text{-}6.9 \times 10^6$
Flow rate (mL/hr)	30	100
Eluent	Ammonium acetate—acetic acid buffer, pH 4.44	Ammonium acetate—acetic acid buffer, pH 4.4
Concentration gradient	0.015-6.0 _M_	0.015-6.0 _M_
Gradient generator	9-chamber[b]	9-chamber[b]

[a] Columns were constructed of stainless steel and jacketed for temperature control.

[b] Two chambers contained 0.015 _M_, two chambers contained 4.0 _M_, and five chambers contained 6.0 _M_ buffer.

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The chromatograms were developed by eluting the sample constituents from the resin column with an ammonium acetate—acetic acid buffer solution (pH 4.4), whose acetate concentration gradually increased from 0.015 to 6.0 M. The uv absorbances of the column effluent were measured at 254 and 280 nm with a dual-beam, flow-through photometer and recorded on a strip chart. Subsequently, the column effluent (eluate) was split so that 90% of the eluate was collected in a fraction collector and 10% was channeled to the cerate-fluorescence detector to measure and record the create oxidizability of the column effluent.

The individual HPLC eluate fractions were pooled to correspond to the chromatographic peaks recorded on the chromatograms of the uv absorbing and cerate-oxidizable constituents. The pooled or collective HPLC fractions representing separated chromatographic constituents were frozen for preservation and further sample testing.

Native fluorescence—Natural or native fluorescence is the property of a compound to emit fluorescence when excited by light. The excitation energy or wavelength of the exciting light and the spectra of the fluorescence or emission light is characteristic of the compound possessing native fluorescence. This property is used to detect compounds separated by HPLC from aqueous effluents of coal conversion processes. Therefore, native fluorescence was evaluated as a detection characteristic for nonvolatile components of wastewater effluents. The commercial fluorometer used as the HPLC detector had an excitation wavelength of 360 nm and measured all emission greater than 400 nm. The uv and fluorescence chromatograms of a concentrate of an unchlorinated secondary effluent (collected 2/21/78, Table 43, Appendix) are given in Figure 4. Direct comparison of the chromatograms is possible because the fluorometer monitored the HPLC eluate immediately after it emerged from the uv detector. Figure 5 shows corresponding chromatograms of the uv and fluorescence chromatograms of another secondary effluent (collected 3/12/79, Table 43, Appendix). The two figures clearly show that fluorescence detects compounds not seen by uv and vice versa. Therefore, native fluorescence was routinely used to enhance detection of constituents separated by HPLC.

Improved HPLC Methodology--

The resolution of HPLC strongly basic anion exchange columns is improved by simply soaking the resin in ethanol prior to and during the column-packing step.[23] This improved HPLC methodology was compared with the HPLC method previously described.[6] A sample of the primary effluent from the Oak Ridge West Wastewater Treatment Plant (collected 12/12/77, Table 42, Appendix) was concentrated 1900-fold. A 0.77-mL aliquot of the concentrate was chromatographed using the former HPLC methodology (i.e., 0.15- by 100-cm column packed with Bio-Rad Aminex A-27 resin, 12 to 15 μm nominal diam) processed by the standard resin treatment.[6] A second 0.72-mL aliquot was chromatographed using the improved HPLC methodology (i.e., a 0.45- by 50-cm column packed with Bio-Rad Aminex A-27 resin, 12 to 15 μm nominal diam).

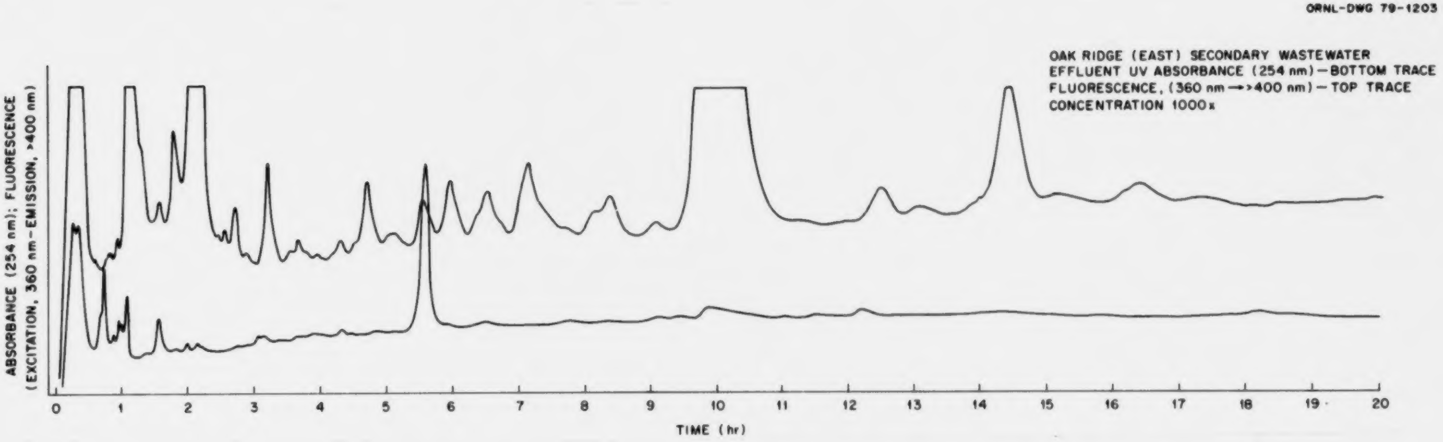

Figure 4. Native fluorescent and uv-absorbing constituents in ORSE secondary effluent.

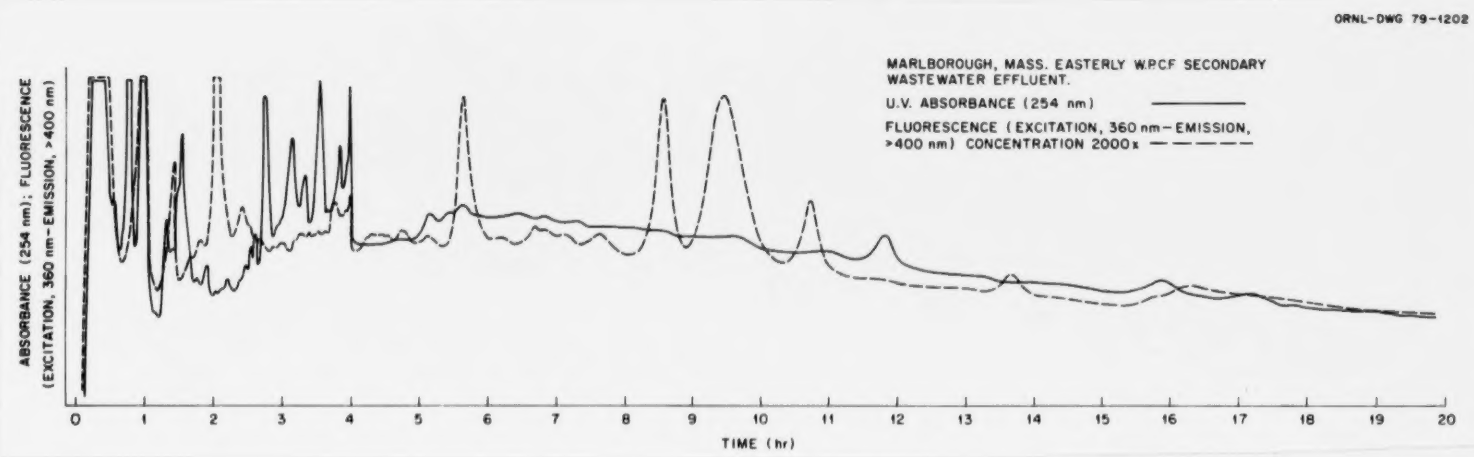

Figure 5. Native fluorescent and uv-absorbing constituents in MMSE effluent.

Chromatograms of both samples shown in Figure 6 show comparable resolution in a much shorter time with the improved method. Consequently, most HPLC runs were made using ethanol treated resin columns.

Characterization and Identification of Constituents

Liquid chromatography of the concentrated effluent samples permitted isolation of fractions from the complex effluent concentrate. The eluate from the preparative-scale HPLC system was collected in fractions representing single peaks or combinations of peaks and proccessed through the following treatment so that the isolated constsituents could be characterized or identified and tested for mutagenic activity: (1) removal of eluent buffer salts, (2) uv spectrometry, (3) gas chromatography, and (4) gas chromatography/mass spectrometery.

Treatment of HPLC Eluate Fractions--

Eluate fractions corresponding to individual eluted chromatographic peaks or combinations of peaks from the HPLC separations were frozen at -60°C in polyethylene bottles and lyophilized at 2 Pa (15 μm Hg) for removal of the eluting solvent (ammonium acetate—acetic acid buffer) from the collected organic fractions. The organic residues were then dissolved in spectroscopic-grade methanol and stored at -20°C for further chemical analysis and mutagenic activity testing. Immediately prior to further testing, the methanol solution was dried under a nitrogen gas blanket using a slow flow of N_2 gas to protect the organics from air oxidation.

Gas Chromatography--

Conversion of the nonvolatile constituents to volatile compounds was necessary for analysis by gas chromatography. The principal method for forming volatile derivatives from the nonvolatile compounds was silylation with bis(trimethylsilyl)-trifluoroacetamide.[6] However, to simplify the interpretation of mass spectra some samples were converted to their methyl esters before gas chromatography and mass spectrometry. Methylation was accomplished with diazomethane in ether solution.[24] The derivatized samples were injected directly onto dual columns of a Tracor model 222 gas chromatograph equipped with a dual-flame ionization detector and dual electrometers. The two columns (0.64-cm OD x 1.8-m Pyrex tubing packed with 3% OV-1 or 3% OV-17 on 80/100-mesh Chromosorb W-HP) were simultaneously programmed from 100 to 325°C at 10°C/min. Inlet and detector temperatures were maintained at 200 and 250°C, respectively, and the helium carrier-gas flow rate was 80 mL-min. Retention times were measured by comparison with the elution of standard hydrocarbons. A typical gas chromatogram is shown in Figure 7.

Gas Chromatography/Mass Spectrometry--

Gas chromatography/mass spectrometry (GC/MS) was used in the chemical characterization and identification phases of this research program. GC/MS was usually performed on aliquots of trimethylsilyl (TMS)-derivatized samples (see previous subsection). The samples were

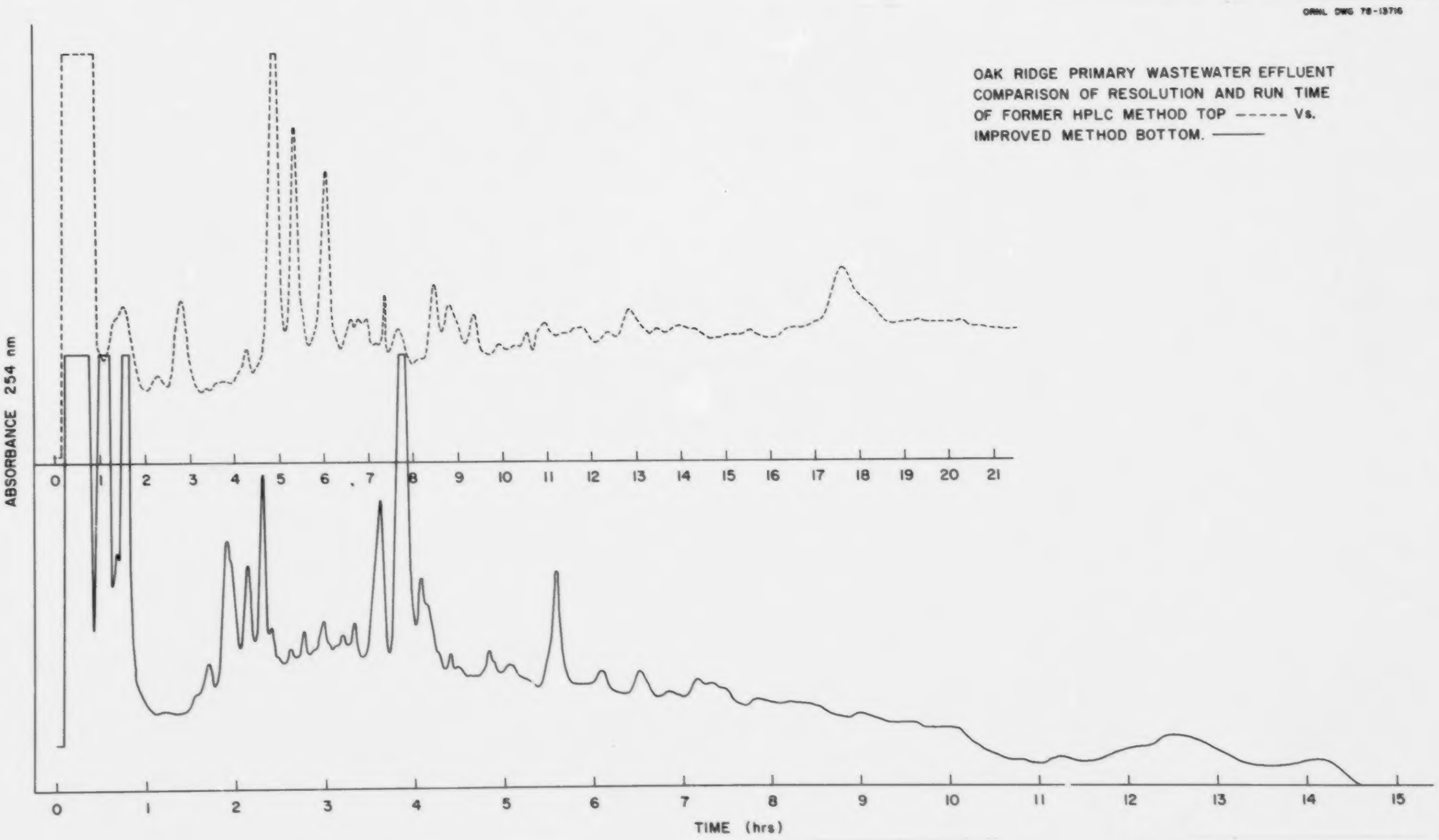

Figure 6. Comparison of chromatograms of a primary effluent resulting from chromatography by the former HPLC method and the improved HPLC method.

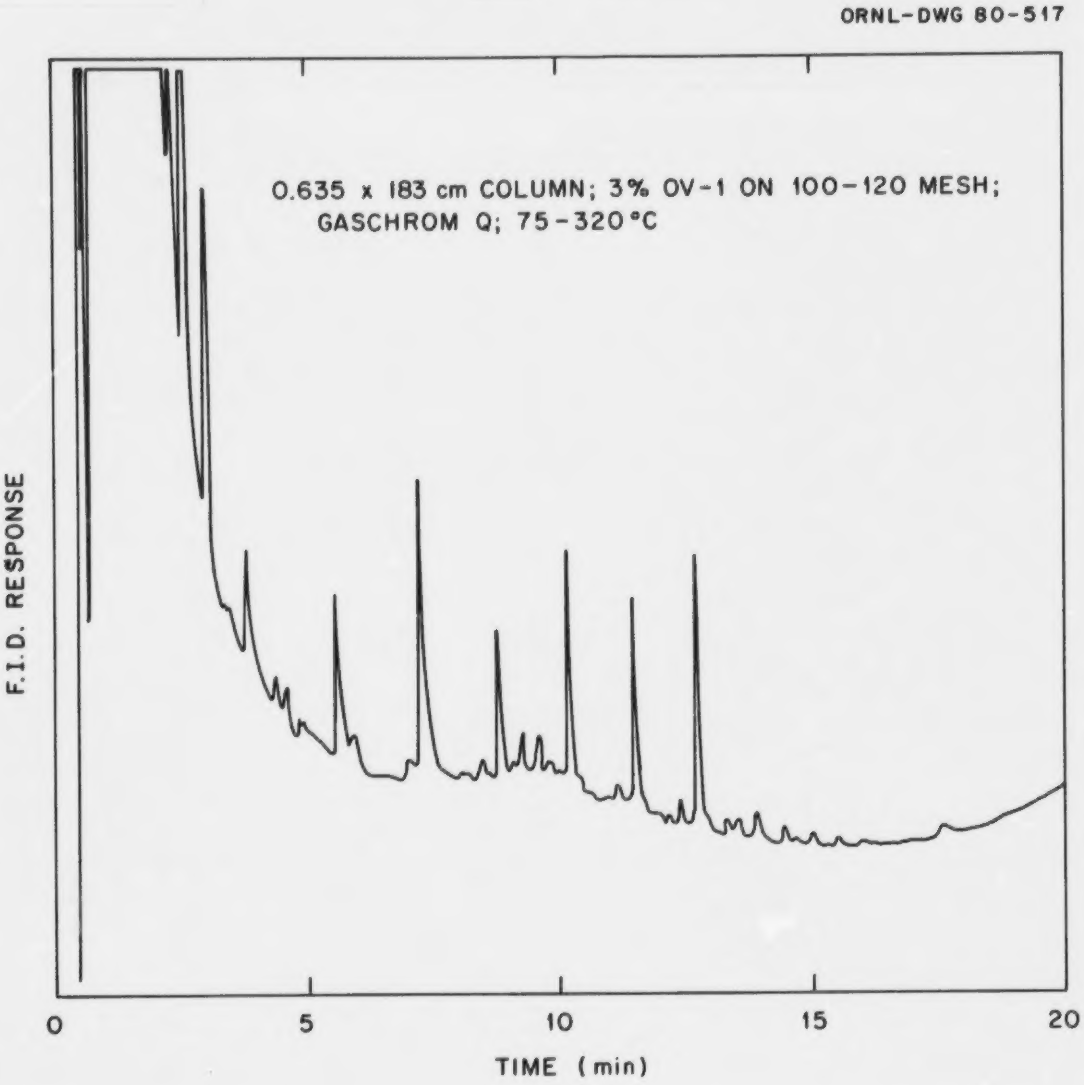

Figure 7. Typical gas chromatogram of HPLC eluate fraction showing flame ionization detector (F.I.D.) response to trimethylsilylated derivatives of organic constituents.

run routinely on a Varian Aerograph Series 1400 gas chromatograph/ Finnigan 3000 peak identifier mass spectrometer interfaced to a System Industries System 150 using a PDP 8/E (Digital Equipment Corp.) computer. Mass spectra were compared by the computer with mass spectra files in the Riber 400 GC/MS Data System Computer Library for quadrupole systems (25,000 spectra) and the EPA/NIH Abbreviated Spectra and Research Library (25,500 spectra) to obtain probable identifications. Mass spectra were also compared manually with several references.[25-27] Matching of the fragmentation pattern for an unknown constituent with that obtained on a reference standards was necessary for absolute identification.

The operating parameters used for GC/MS were (1) gas chromatography using a Pyrex glass column (0.64-cm OD x 1.8 m) packed with 3% Dexsil 300 on Anakrom Q, 90-100 mesh; helium flow rate of 20 mL/min; columns programmed from 80 to 320°C at 10°C/min; injector temperature, 220°C; and (2) mass spectrometry using manifold temperature, 75°C; ionizing electron energy, 70 eV; mass scan range, 40-490; integration time, 5 ms; sample/amu, 1; total run time, 25 min; vacuum, 1.3×10^{-5} Pa (10^{-7} torr). Daily calibration of the mass range was performed by introduction of a minute amount of perfluorotributylamine (FC-43 mass number standard).

DISINFECTION TREATMENT OF EFFLUENT SAMPLES

During the initial phase of this research program ozonation and uv irradiation were conducted in the laboratory on grab effluent samples to investigate the effects of these processes on the chemical composition of the effluent. Subsequent samples collected during the field testing phase of the program were grab samples of disinfected effluent collected at the treatment plants during the course of their normal operation. All chlorinated effluent samples were grab samples of disinfected effluent collected following routine chlorination of the plant effluent.

The laboratory methods for ozonation and uv irradiation are as follows.

Ozonzation

Wastewater effluent samples were treated with ozone generated from a Wellsbach T-23 ozonator. A small fraction of the oxygen feed gas was converted to ozone, which was allowed to react with the sample for a predetermined amount of time, depending on the total dose desired. The reaction vessel was a Pyrex round-bottomed flask using a medium-porosity gas diffuser to disperse the ozone and the oxygen carrier gas through the magnetically stirred sample solution. Unconsumed ozone was trapped in a 20% KI solution during the reaction run; after the run, the headspace above the effluent sample was purged with air through the trap. The KI solution was analyzed iodometrically to determine the amount of unconsumed ozone.[28] A wet-test meter recorded all gas flows. A schematic drawing of the ozonation equipment is shown in Figure 8.

Ozone concentration in a gas was determined by bubbling the gas through a 20% KI solution and titrating the liberated I_2 with sodium thiosulfate in an acidic medium with starch indicator.[28] A typical reaction run had a gas concentration of 25 mg O_3 per liter of gas and a gas flow of 0.25 L/min. For example, with a 4.5-L effluent sample and a reaction time of 8.64 min, the resulting dosage was 12 mg O_3/L effluent.

Ultraviolet-Light Irradiation

Samples of primary or secondary wastewater effluents were exposed to varying dose levels of uv irradiation by pumping the effluent sample through a uv sterilizer. A model H-1.0 uv sterilizer with sight port (Pure Water Systems, N.J.; see Figure 9) and an LFE centrifugal pump (Eastern Industries Div., 1 L/min max.) were used. The internal volume of the baffled-flow cell was 370 mL. A quartz tube with a low-pressure mercury lamp, capable of emitting uv irradiation at 254 nm, was positioned longitudinally through the center of the cell. As the sample flowed through the cell, it was exposed to a constant uv-irradiation dose level. The amount of energy impinging on the sample was calculated by multiplying the retention time in the cell by the measured optical irradiation intensity.

The retention time of a sample at each flow rate was calculated using the following equation:

$$\frac{V_c}{F} = t_R,$$

where

V_c = volume of cell,

F = flow rate,

t_R = retention time.

The t_R value times the optical irradiation measured with an IL-700 research radiometer and photometer gave the amount of energy impinging on the sample during this time ($\mu W \cdot s/cm^2$).

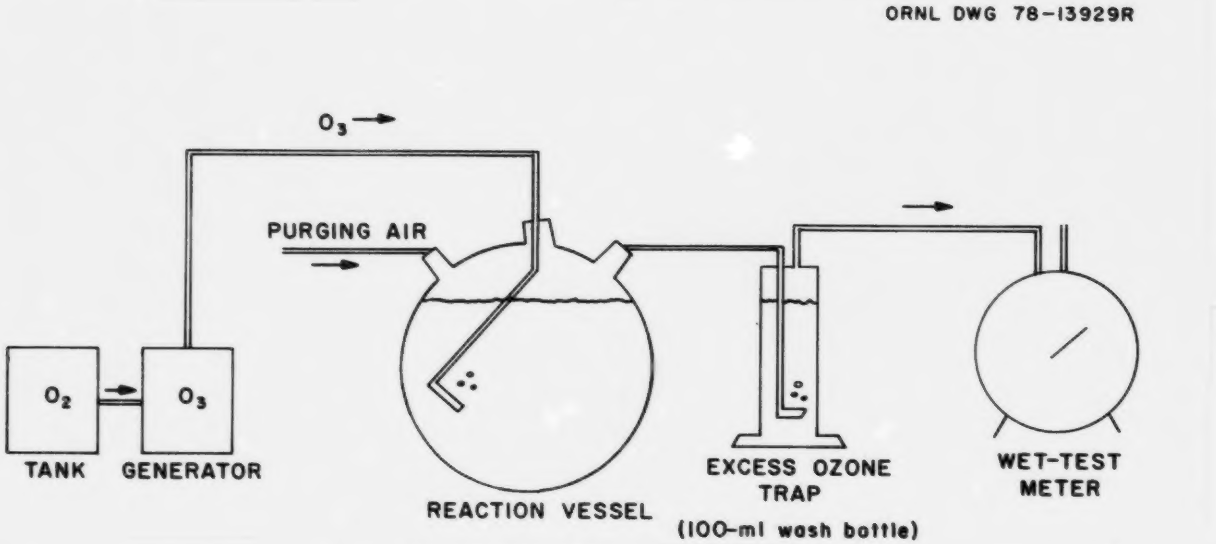
ORNL DWG 78-13929R
O_3 →
PURGING AIR
→
O_2
O_3
TANK
GENERATOR
REACTION VESSEL
EXCESS OZONE TRAP
(100-ml wash bottle)
WET-TEST METER

Figure 8. Schematic drawing of ozonation equipment.

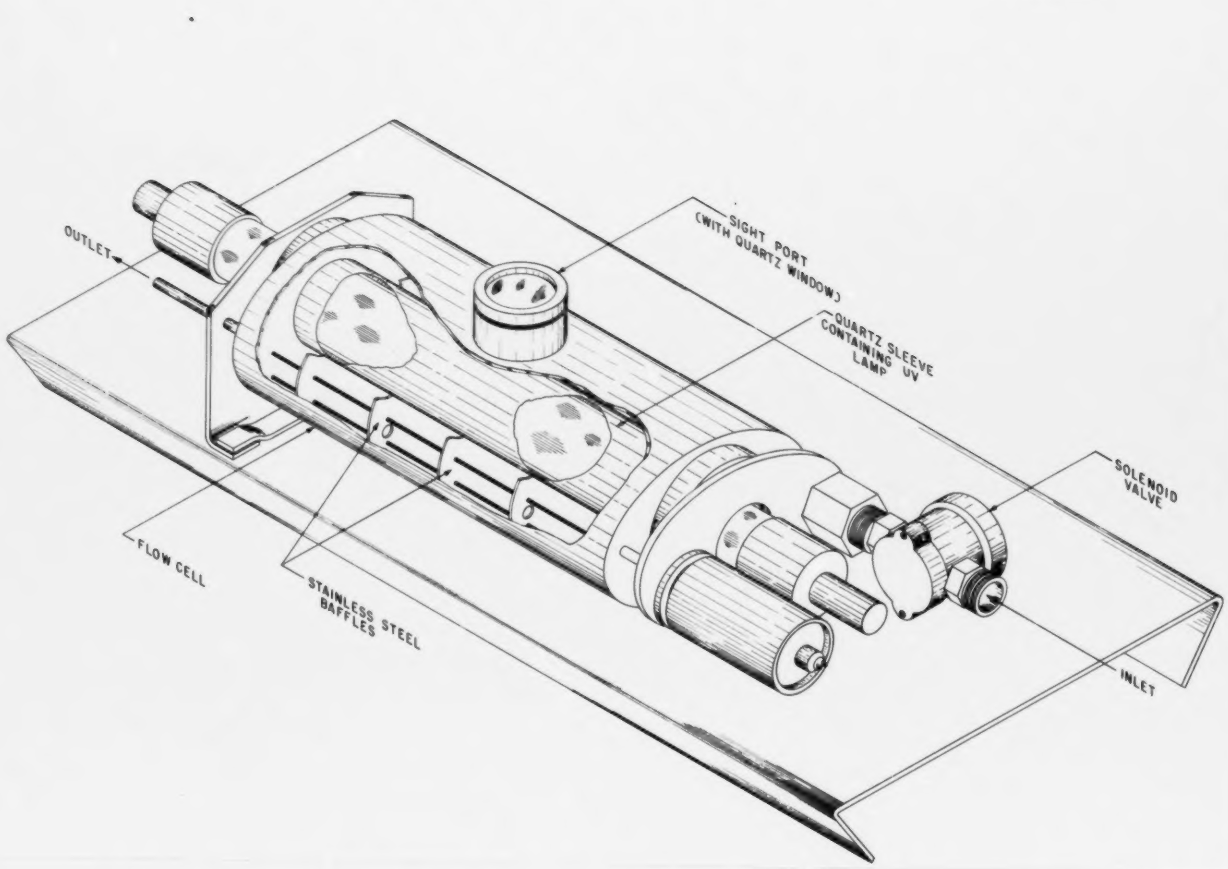
ORNL DWG 78-13939R
SIGHT PORT (WITH QUARTZ WINDOW)
QUARTZ SLEEVE CONTAINING UV LAMP
SOLENOID VALVE
OUTLET
FLOW CELL
STAINLESS STEEL BAFFLES
INLET

Figure 9. Flow cell (uv sterilizer) for irradiation of wastewater effluents.

DETERMINATION OF MUTAGENIC ACTIVITY

Two test systems have been used to assay the mutagenic potential of wastewater effluent concentrates and fractions derived from them. The first of these involves various bacterial mutagenicity tests which are designed to assay mutagenic potential in a nonmammalian system of high resolution.[18,20] The other, in vivo unscheduled DNA synthesis in mice, is a mammalian system designed to determine if the fraction tested or any of its metabolites reach the germ cells and damage DNA in these cells.[16,17]

Bacterial Test Systems

Reversion mutational assays in several strains of Escherichia coli and Salmonella typhimurium were used to test effluent concentrates and HPLC eluate fractions for mutagenic activity. The tests involved standard techniques[18-20] in which nutrient broth was inoculated with the frozen stock culture and grown overnight until the density had reached approximately 1 to 2 x 10^9 viable cells per milliliter. A fresh cell suspension was used each day for the tests. Checking of tester strain genotypes with regard to confirming histidine requirement, deep-rough character, and sensitivity to ultraviolet light and ampicillin was performed along with each test to ensure the bacteria had not acquired amomalous characteristics. Stock cultures were also checked periodically to ensure that close to 100% of the bacteria contain the R factor. Each strain used was run with the recommended strain-specific positive mutagenic control compounds; for example, the following were used with the TA strains.

Strain	Compound
TA-1535/TA-100	Methylmethanesulfonate (MMS) and Ethylmethanesulfonate (EMS)
TA-1537/TA-1538/TA-98	Hycanthone methanesulfonate (HMS)

A minimum of two plates per dose level was used and when positive results were obtained, the test was repeated as long as time permitted. In the early stages of this study, many tests were performed in the presence and absence of the 9000 g supernate of liver homogenate from Aroclor-induced rats (S-9). This was done to check for the presence of compounds which require mammalian activation in order to become mutagens. Since S-9 did not enhance the mutagenicity of any concentrates or fractions in these early tests, its use was discontinued in later testing. Because of problems that developed approximately halfway through the research program regarding degradation of the bacterial test cells despite periodic check of the bacterial cultures, very stringent precautions were taken thereafter regarding quality assurance of the bacterial strains and stringent filtrations of the sample material to avoid sample contamination. The following were performed for each sample tested: (1) filtration (through Swinex filters) and testing in TA-1535 and TA-1538, (2) no filtration and testing in *Salmonella* strain TA-1535, and (3) no filtration and observation in a test without the addition of bacteria but in the presence of histidine and biotin. Plates were incubated at 37°C for 48-72 hr before the scoring of revertants.

E. coli strains WP-2 (tryptophan requiring) and WU-3610 (leucine and tyrosine requiring) have been used. The principal bacterial tester strains used were the *Salmonella* strains TA-1535, TA-1537, TA-1538, TA-100, and TA-98. Strains TA-1535 and TA-100 respond to base pair substitution mutagens, and TA-1537, TA-1538 and TA-98 respond to frame-shift mutagens. Strains TA-100 and TA-98 contain an R factor which makes them much more sensitive to some types of mutagens. Using these strains, other investigators[29] have obtained positive results (mutagenic activity) with drinking water concentrates. Another *Salmonella typhimurium* strain, SV3, was also tested. The normal arabinose metabolic pathway is blocked in the SV3 strain and, consequently, they do not survive in arabinose-containing media. Forward mutations in SV3 will permit them to utilize arabinose and, consequently, survive in arabinose media.[30,31] It was concluded from comparative results with TA strains that SV3 did not offer any advantage.

Presentation of Bacterial Mutagenicity Data--

To simplify data presentation, the mutagenicity results have generally been presented in a summary fashion as follows: tests that showed less than two times the activity of contemporary negative controls are presented as "negative" (-), those which showed from two to five times the activity of the negative controls are called "weakly positive" (±), and those which showed more than five times the activity of negative controls are considered "positive" (+). In some cases, data has been presented as mutated revertant colonies per unit of volume or mass. For the chromatographic fractionated samples, data are presented in ratios (e.g., 24/24) representing the number of positive, slightly positive or negative tests relative to the number of HPLC fractions.

In Vivo Unscheduled DNA Synthesis Test

This test, which was developed at ORNL,[16,17] is an in vivo test that measures the incorporation of tritiated thymidine into spermatids of treated mice. These cells normally do not synthesize DNA and are stimulated to do so only when the DNA is damaged. The test is relatively simple and very sensitive. It measures the capacity of a potential mutagen to reach germ cells and damage DNA in these cells.

Groups of male mice are given intraperitoneal injections of suitable solutions of effluent concentrates or selected constituents.

Simultaneously, or at various times after treatment, the animals are given testicular injections of tritium-labeled thymidine, [^{3}H]dThd. Control groups receive only [^{3}H]dThd in isotonic saline solution. Lethal doses of the test material must first be determined by means of toxicity studies so that experimental exposure levels can be established.

Sixteen to seventeen days after treatment, the animals are killed and the sperm from treated and untreated (control) animals are purified to obtain only sperm heads. These sperm heads are derived from early spermatid stages. Comparisons of the unscheduled incorporation of [^{3}H]dThd in the sperm of the test group vs the control group give a measure of the damage to the DNA of the germ cells induced by the test material. The activity of [^{3}H]dThd in the sperm heads is measured by liquid scintillation counting.[16,17]

SECTION 6

CHEMICAL EFFECTS OF DISINFECTION (LABORATORY STUDIES)

Initial procedure evaluation and comparative studies were conducted with effluent samples from the Oak Ridge East and West wastewater treatment plants.

The objectives were to evaluate disinfection procedures on a laboratory scale and to make initial comparative studies of the chemical effects on organic compounds. These effects were detected by changes in HPLC chromatographic profile between the nondisinfected effluent (control) and the disinfected effluent. No laboratory-scale chlorination studies were performed.

OZONATION

Initial efforts were directed toward establishing parameters for ozonation of secondary effluents. In the secondary effluent of the Oak Ridges East Wastewater Treatment Plant (collected 5/18/77, Table 43, Appendix), an ozone dose of approximately 14 mg of ozone per liter of effluent was required for a >99.99% bacterial kill (Figure 10) as established by the standard plate-count method for determination of bacteria.[32] Determinations of the bacterial quality of the control and treated water samples were made using this method; however, to facilitate comparison with other disinfection studies, later determinations used the total coliform method (membrane filter).[33]

The reaction conditions, chemical data, and disinfection data of the ozonation experiments used to calculate the kill curve in Figure 10 are given in Table 4. The chemical oxygen demand (COD) and BOD of the samples trend downward with increasing dose. The total organic carbon (TOC) apparently increases slightly, then decreases with increasing dose. No conclusion can be drawn regarding total Kjeldahl nitrogen (TKN).

Ozonation - HPLC Study 1

Large differences were detected between high-pressure anion exchange chromatograms of concentrates of unozonated secondary wastewater effluent (collected 4/11/77, Table 43, Appendix) and those which had been ozonated for 3-, 15-, and 30-min periods with an ozone gas concentration of 6 mg per liter of oxygen gas. This treatment resulted

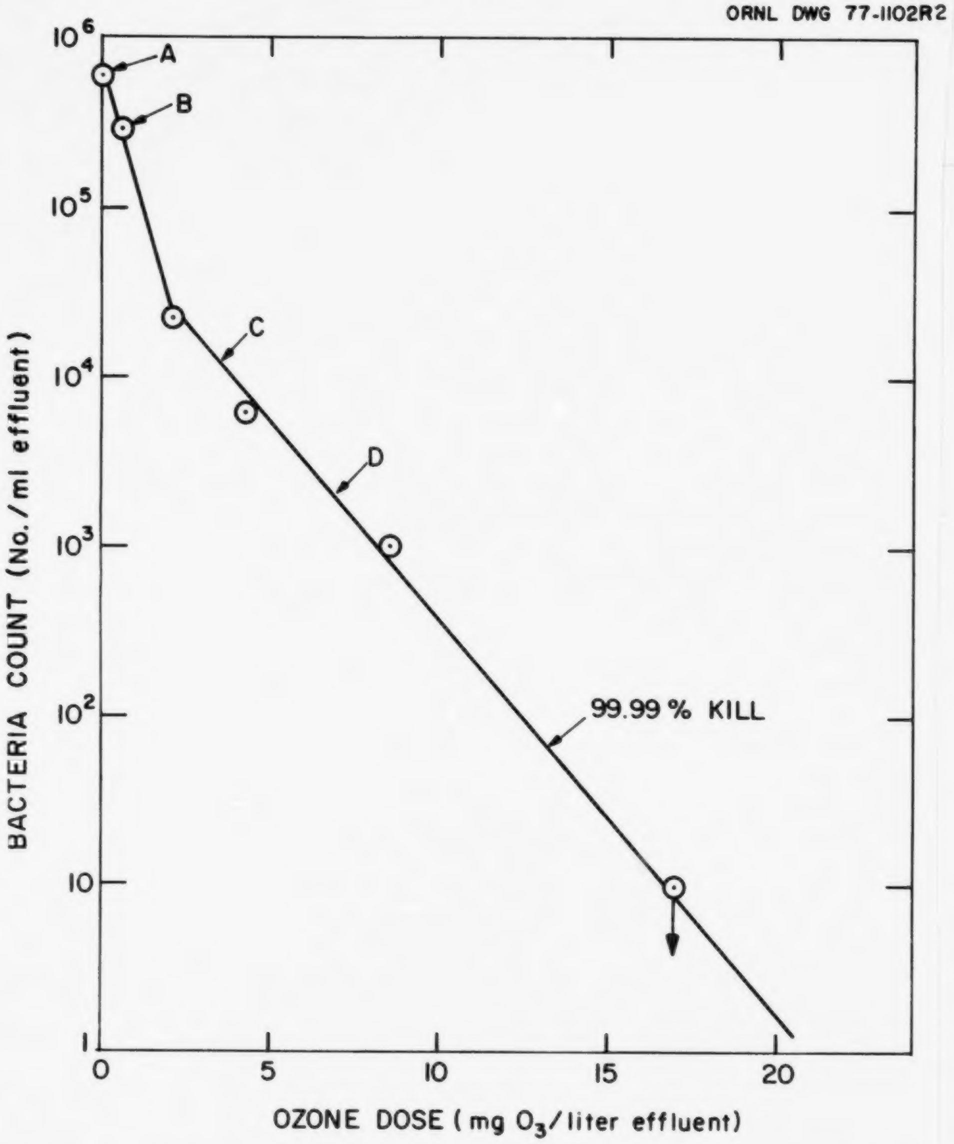

Figure 10. Disinfection of secondary effluent with ozone. Bacteria counts were made using the standard plate-count method.[32] The letters A, B, C, and D indicate disinfection levels of experiment HPLC study 1.

TABLE 4. OZONATION OF FILTERED SECONDARY EFFLUENT FROM THE OAK RIDGE EAST WASTEWATER TREATMENT PLANT: CHEMICAL AND DISINFECTION DATA

Reaction time[a] (min)	Total ozone dose (mg/L of effluent)	Bacteria count[b] (No./mL)	pH	Concentration (mg/L)			
				COD	BOD_5	TKN	TOC
Control	0.0	600,000	7.5	24	9	20.0	7.9
5	0.7	300,000	7.8	26	9	18.6	7.6
15	2.1	22,500	7.8	20	7	18.6	8.3
30	4.3	6,100	7.9	19	<5	19.0	8.6
60	8.5	1,000	7.9	20	<5	23.5	7.5
120	17.1	<1	7.9	16	<5	18.2	7.2

[a] Room temperature (approximately 25°C); ozone-oxygen gas mixture at 0.08 L/min containing 8.9-mg ozone per liter of gas; 5-L effluent sample; glass-frit sparger with magnetic stirrer.

[b] Standard plate-count method.[32]

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in total doses of 0.7, 3.5, and 6.9 mg of ozone per liter of wastewater effluent. The chromatograms of the uv-absorbing and the cerate-oxidizable constituents in the control sample and the ozonated samples are shown in Figures 11 and 12. The ozone dosages of these samples are labeled A, B, C, and D on Figure 10 for comparative purposes to show approximate levels of disinfection, but bacterial counts were not made for these samples.

The following hypothesis is proposed to explain the changes in HPLC constituents that occurred during ozonation. Ozone initially reacts with organic constituents that are probably of a higher molecular weight and are not separated in this chromatographic procedure, although some may be associated with the first chromatographic peak. The higher-molecular-weight constituents are broken down into smaller-molecular-weight fragments, which are separated chromatographically and detected as uv-absorbing and oxidizable peaks. This is indicated by a decrease in size of the first peaks (Figures 11 and 12) with increasing ozone dosage and an increase of poorly resolved peaks (and apparent column overloading) in the middle of the chromatograms for the intermediate dosages. As ozonation proceeds, these fragments are, in turn, oxidized and decomposed, as shown in Figures 11 and 12, for the highest dosage.

Ozonation - HPLC Study 2

Figure 13 shows the chromatograms of uv-absorbing and cerate-oxidizable constituents in another ozonation experiment with secondary effluent from the Oak Ridge East Wastewater Treatment Plant (collected 8/9/77, Table 43, Appendix). Two 6-L aliquots of Oak Ridge East Wastewater Treatment Plant secondary effluent were ozonated in the laboratory to dosages of 3.5 and 20.0 mg ozone per liter of wastewater. Reaction conditions were 25°C; ozone gas concentration, 19.4 mg ozone per liter of gas (oxygen); 12-L glass reaction flask; magnetic stirrer; porous glass-frit gas diffuser; ozone-oxygen gas mixture flow rate of 250 mL/min.

From the chromatograms of the uv-absorbing constituents in 2000-fold concentrates of the ozonated effluents, it may be inferred that most of the destruction of these compounds occurs rapidly and within the first 3.5-mg/L effluent dosage of ozone. With respect to the oxidizable constituents, there is an apparent initial destruction of some compounds and an increase in numbers and concentration of others with increasing ozone dosage (e.g., the increase in numbers and size of peaks in the 5- to 15-hr elution period). Chemical data for the control and ozonated wastewater samples are given in Table 5. Note that the disinfection data were not run. The wastewater samples are representative of effluent from an activated-sludge secondary treatment plant. The wastewater was principally domestic, containing very little industrial waste.

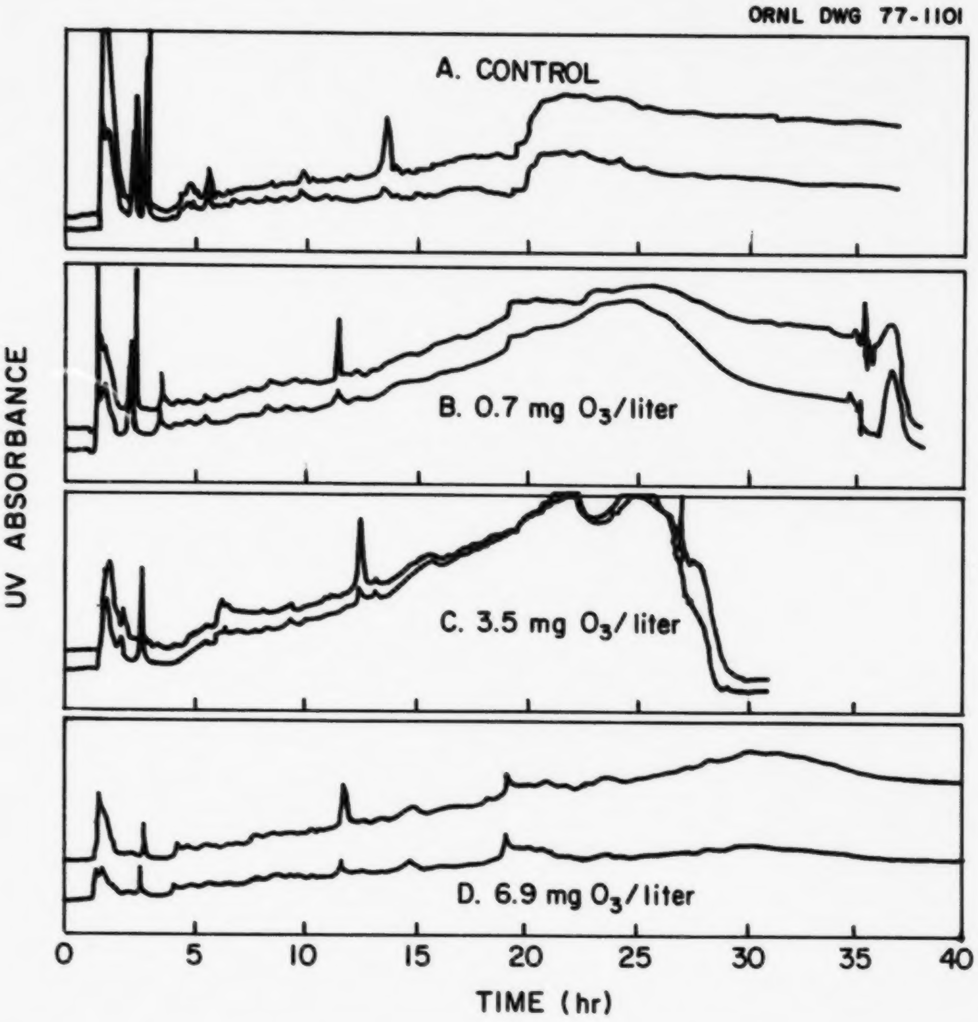

Figure 11. Comparison of uv-absorbing constituents in secondary effluent sample with samples treated with ozone. Sample A is the unozonated control. Samples B, C, and D were ozonated at ozone doses of 0.7, 3.5, and 6.9 mg/L, respectively. Chromatograms were determined for 1.0-mL aliquots of 2500-fold concentrates using standard uv-analyzer chromatographic procedure. For each chromatogram, the bottom and top tracings represent the uv absorbance at 280 nm and 254 nm, respectively. (Ozonation - HPLC Study 1).

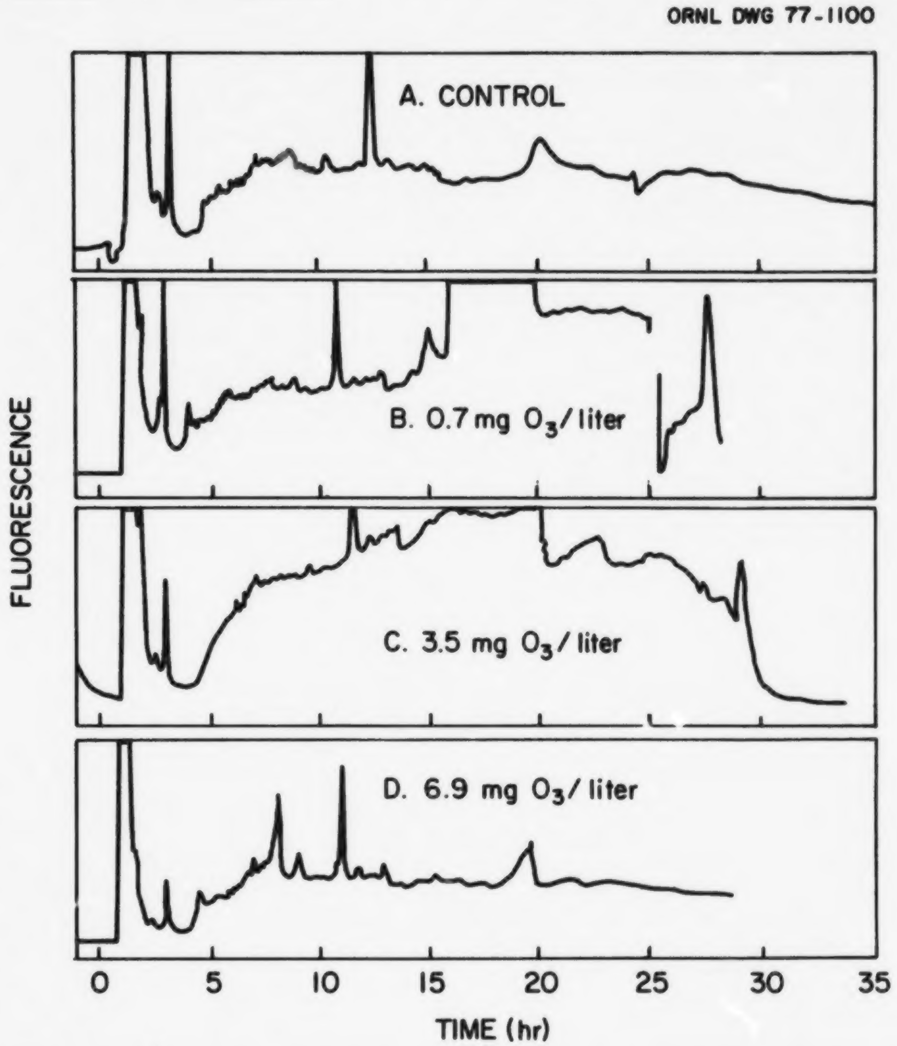

Figure 12. Comparison of oxidizable constituents in a secondary effluent sample with samples treated with ozone. Sample A is the unozonated control. Samples B, C, and D were ozonated with ozone doses of 0.7, 3.5, and 6.9 mg/L, respectively. Chromatograms were determined for 1.0-mL aliquots of 2500-fold concentrates using standard cerate-oxidimetry chromatographic procedure (Ozonation - HPLC Study 1).

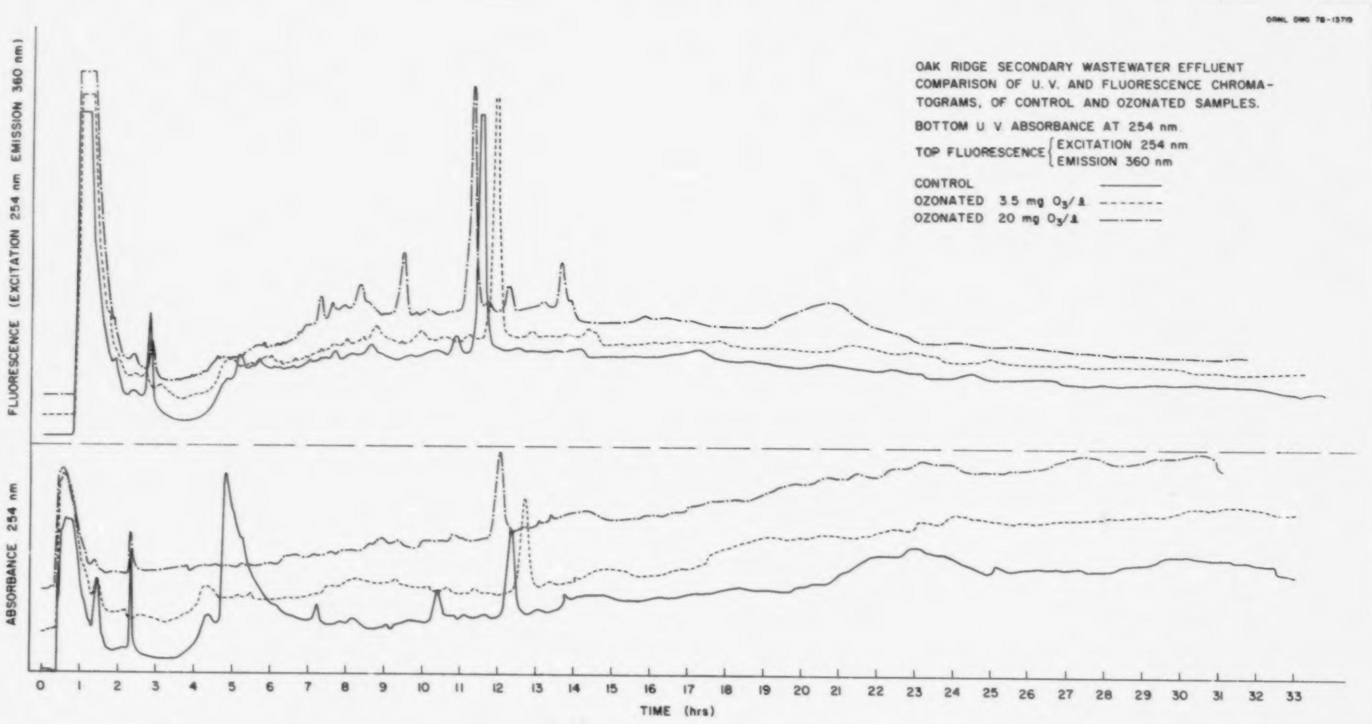

Figure 13. Chromatograms of uv-absorbing constituents and cerate-oxidizable constituents in 2000-fold concentrates of secondary effluent ozonated with 0, 3.5, and 20 mg O_3 per liter effluent (Ozonation - HPLC Study 2).

TABLE 5. OZONATION OF FILTERED SECONDARY EFFLUENT FROM THE OAK RIDGE EAST WASTEWATER TREATMENT PLANT: CHEMICAL DATA (OZONATION - HPLC STUDY 2)

Reaction time[a] (min)	Total ozone dose (mg ozone/L of effluent)	pH	Concentration (mg/L)			
			COD	BOD_5	TKN	TOC
Control	0.0	7.61	30	6	18.2	10.8
4.3	3.5	7.69	28	7	17.4	9.8
24.8	20.0	7.84	24	5	17.2	10.5

[a] Room temperature (about 25°C); ozone-oxygen mixture at 250 mL/min containing 19.4 mg ozone/L gas; 6-L effluent sample; glass-frit sparger with magnetic stirrer.

Ozonation - HPLC Study 3

An experiment using a typical primary effluent was conducted in order to increase the possibility of detecting chemical changes on organic constituents. The higher concentrations of organic components in primary effluent should permit easier detection of chemical effects caused by the disinfection method. Subsequent uv-irradiation and chlorination experiments were conducted on the same effluent for comparative purposes.

In this experiment, a 21-L sample of Oak Ridge West Wastewater Treatment Plant primary effluent (collected 12/12/77, Table 42, Appendix) was ozonated in two 10.5-L batches to a dosage of 6.9 mg ozone per liter of effluent. The ozone-oxygen gas flow rate was 0.11 L/min, and the total reaction time was 29.25 min. The system was purged with approximately 1 L of air after each ozonation. In batch 1, the total dose was 72.5 mg of ozone; 26.5 mg of ozone was trapped in the potassium iodide solution following the reaction train, for a reaction or ozonation efficiency of 63%. In the second batch, the total ozone dose was 72.4 mg; 29.6 mg of ozone was measured in the potassium iodide solution following the reaction train, indicating an ozone utilization or a reaction efficiency of 59%. Thus an average experimental equipment. The chromatograms of uv-absorbing constituents in the untreated (control) sample and those in the ozonated primary effluent are compared in Figure 14. It is evident that many of the uv-absorbing constituents are destroyed by ozonation. Note, however, the formation of one major peak which elutes at approximately 7 hr.

ULTRAVIOLET-LIGHT IRRADIATION

Ultraviolet-light irradiation is being considered as an alternative disinfection method to chlorination and ozonation; therefore, studies were conducted to determine the chemical and biological effects on nonvolatile organics which result from this treatment. The method involved irradiating untreated secondary effluent at a fixed optical irradiance ($\mu W/cm^2$) for different flow rates (i.e., different retention times within the uv flow cell).

Total coliform (membrane-filter method[33]) survival curves were determined for uv irradiation of typical primary and secondary effluents from the Oak Ridge wastewater treatment plants (Figure 15). The uv-irradiation dosage and total coliform survival data for this series of experiments are given in Table 6 for the secondary effluent (collected 7/13/78, Table 43, Appendix) and in Table 7 for the primary effluent (collected 7/17/78, Table 42, Appendix). For the untreated primary effluent, the BOD_5 was 80 mg/L and the pH was 7.5. The untreated secondary effluent BOD_5 was 19 mg/L and the pH was 7.1. The survival curves were used to establish the parameters for subsequent irradiation experiments.

The effects of uv-irradiation on the chemical composition were evaluated in a series of analyses. Samples of unchlorinated secondary effluent were collected at the Oak Ridge East Wastewater Treatment Plant (12/21/78, Table 43, Appendix) and were delivered immediately with no refrigeration to the laboratory. Ambient temperature during collection and transportation was approximately 0°C. All samples were filtered through an 8-μm Millipore filter, and pumped through the uv sterilizer at different flow rates to vary the irradiation level. A 20-L sample from each irradiation level was lyophilized, concentrated 2000-fold, and analyzed.

Chemical characterization and disinfection data for the control and uv-irradiated samples are given in Table 8. The TOC and TKN appear to decrease as the uv exposure increases. However, the HPLC chromatograms of the same samples show no significant change in composition (Figure 16).

COMPARISON OF CHLORINATION, OZONATION, AND UV-IRRADIATION EFFECTS

Secondary Effluent

Sixty liters of unchlorinated secondary effluent and 20 L of chlorinated effluent (2-mg/L chlorine dose; residual chlorine, 0.5

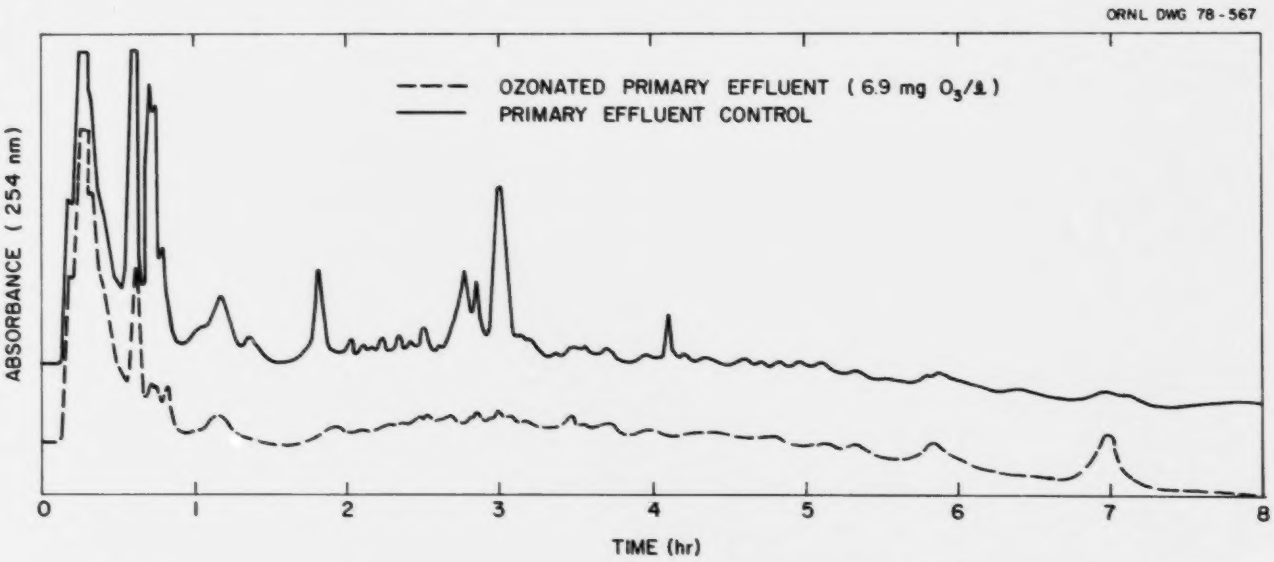

Figure 14. Chromatograms of uv-absorbing constituents in ozonated primary effluent (ozone dosage, 6.9 mg/L) compared with undisinfected control primary effluent. Samples were collected from the Oak Ridge West Wastewater Treatment Plant and the ozonated sample was ozonated in the laboratory; ozonated sample, 0.77 mL of 790-fold concentrate; unozonated sample, 0.77 mL of 700-fold concentrate (Ozonation - HPLC Study 3).

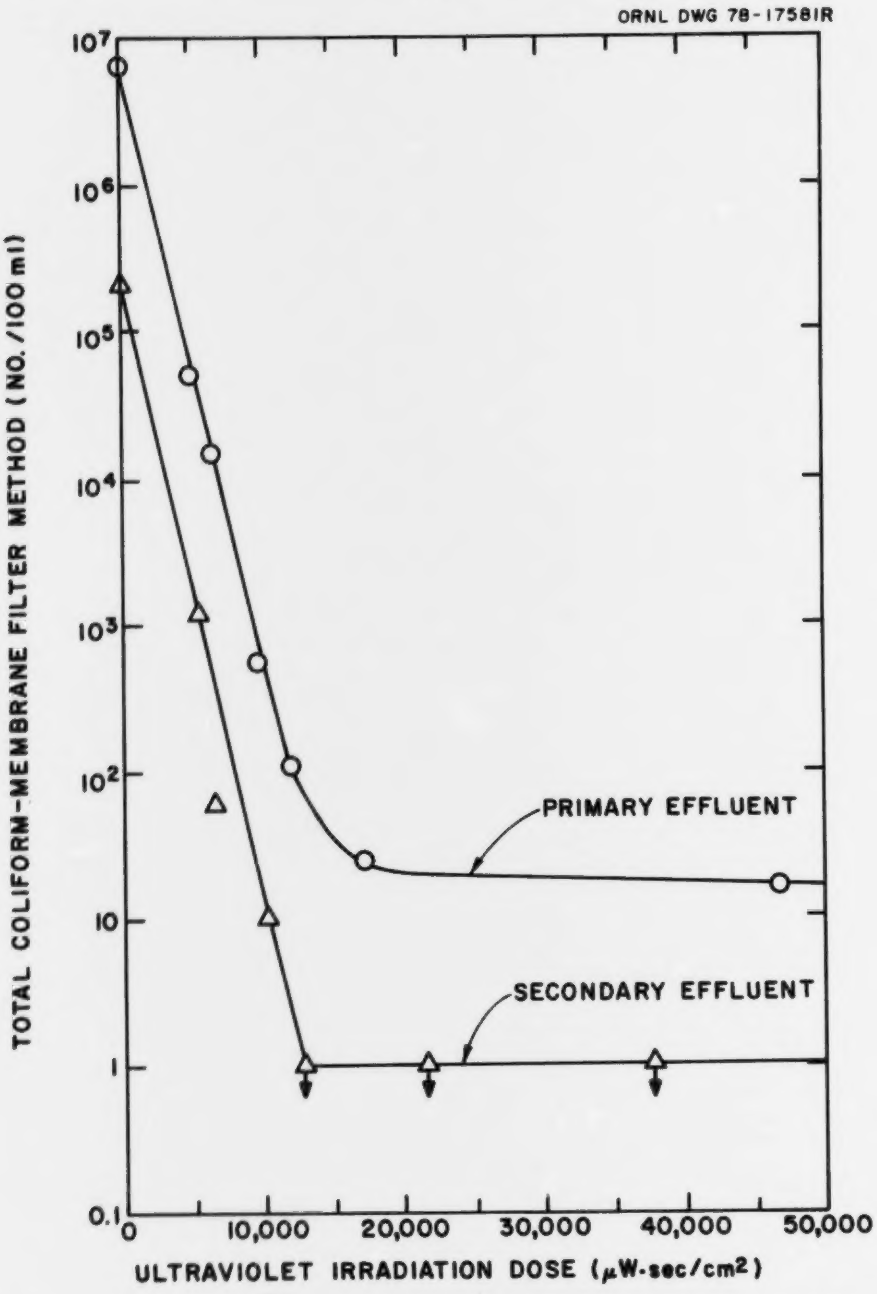

Figure 15. Disinfection of primary and secondary effluent with uv irradiation. Total coliform measurements were made using the membrane-filter method.[33] Because one coliform per 100 mL was the lower limit of detection the last three data points for the secondary effluent are shown as downward pointing arrows (no coliform were detected in the measurements).

TABLE 6. ULTRAVIOLET IRRADIATION, COLIFORM SURVIVAL DATA FOR SECONDARY EFFLUENTS

Sample	Flow (mL/min)	Flow (mL/s)	Retention time, T_R (s)	Irradiation intensity, I (W/cm^2)	Irradiation dosage, E (μW·s/cm^2)	Total coliform[a]	Coliform survival (%)
Control						204,000	100
2	882	14.7	25.2	2.26×10^{-4}	5,690	1,300	0.64
3	732	12.2	30.3	2.17×10^{-4}	6,580	60	0.029
4	481	8.0	46.2	2.18×10^{-4}	10,100	10	0.005
5	395	6.6	56.2	2.26×10^{-4}	12,700	0	0
6	217	3.6	101.9	2.20×10^{-4}	22,400	0	0
7	159	2.7	139.1	2.72×10^{-4}	37,600	0	0

[a] Membrane-filter method.[33]

TABLE 7. ULTRAVIOLET IRRADIATION, COLIFORM SURVIVAL DATA FOR PRIMARY EFFLUENTS

Sample	Flow (mL/min)	Flow (mL/s)	Retention time, T_R (s)	Irradiation intensity, I (W/cm^2)	Irradiation dosage, E (μW·s/cm^2)	Total coliform[a]	Coliform survival (%)
Control						66.5×10^5	100
2	944	15.7	22.5	2.10×10^{-4}	4,960	51,100	0.077
3	722	12.0	30.8	2.15×10^{-4}	6,610	16,100	0.024
4	491	8.2	45.2	2.12×10^{-4}	9,590	590	0.0009
5	398	6.6	55.9	2.11×10^{-4}	11,800	110	0.00017
6	270	4.5	82.4	2.08×10^{-4}	17,100	25	0.00004
7	104	1.7	212.7	2.20×10^{-4}	46,800	17	0.00003

[a] Membrane-filter method.[33]

TABLE 8. EFFLUENT DATA FOR ULTRAVIOLET-IRRADIATION EXPERIMENT

Sample	Concentration factor	UV-irradiation exposure ($\mu W \cdot s/cm^2$)	Total coliform[a] (No./100 mL)	pH	Concentration (mg/L)			
					BOD_5[b]	COD[b]	TOC[b]	TKN[b]
1	1905	Control (0)	200,000	7.4	15	25	20	4.0
2	2000	13,000	<1	7.3	<5	24	20	3.7
3	1818	18,700	<1	7.4	<5	23	19	3.6
4	1800	39,400	40[c]	7.4	<5	22	16	3.3
5	1667	61,200	<1	7.3	<5	23	14	3.3

[a] Membrane-filter method.[33]

[b] Filtered.

[c] This count may represent possible contamination.

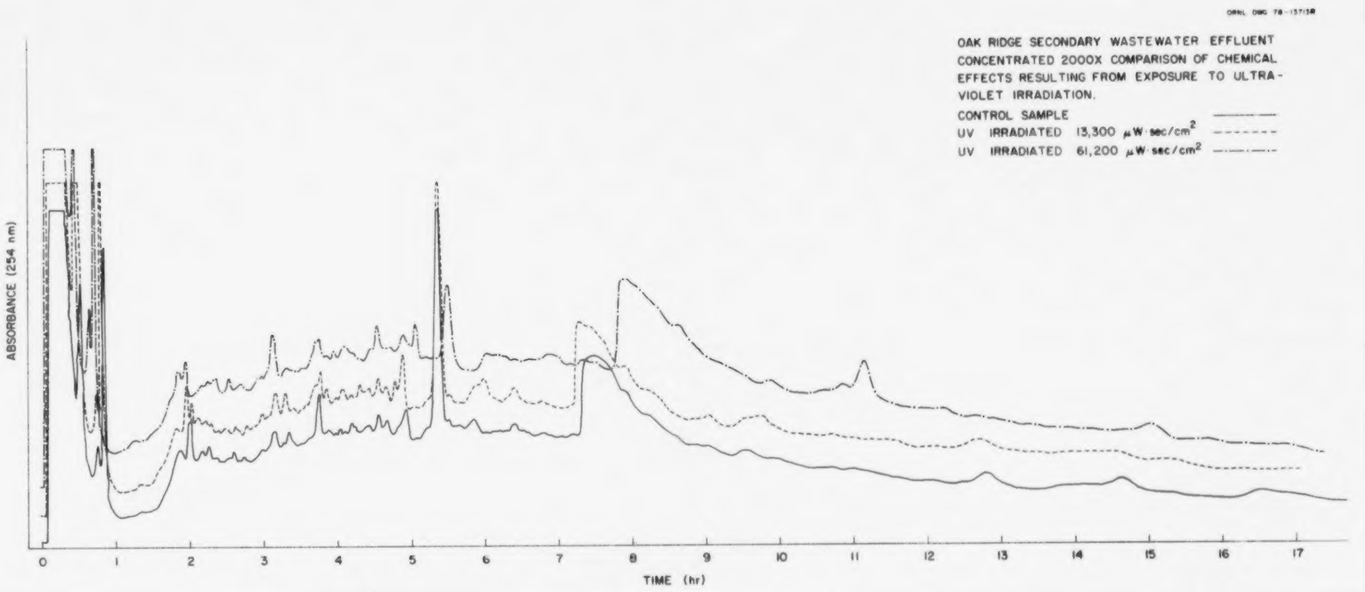

Figure 16. Comparison of chromatograms of uv-absorbing constituents in secondary effluents irradiated with ultraviolet light at 0, 13,300 and 61,200 μW·s/cm^2.

mg/L) were collected from the Oak Ridge East Wastewater Treatment Plant (10/25/77, Table 43, Appendix). The secondary effluent samples were filtered through Whatman No. 2 filter paper. The unchlorinated sample was divided into three 20-L aliquots. One 20-L aliquot was used as the control; the second was ozonated at 25°C with a 8.0 mg/L dosage (76% consumption); and the third aliquot was uv irradiated at 21,200 μW·s/cm^2 and 25°C. Chemical characterization and disinfection efficiencies of these samples are given in Table 9. Reduction in the TOC of the three disinfected samples is apparent and there is an inexplicable increase of the TKN in these samples.

The samples were lyophilized and the concentrates were analyzed by HPLC using both the uv and the fluorescence detectors. Figures 17 and 18 show the chromatograms of these analyses.

Confirming previous effects, ozonation at disinfection concentrations destroyed many nonvolatile organic constituents and produced others, especially oxidizable components. Ultraviolet irradiation apparently had little effect on the organic composition although the peak at 6 hr (Figure 17) appears to be produced by both uv irradiation and chlorination. As also evidenced in previous experiments, chlorination destroys some uv-absorbing and oxidizable constituents and forms several others.

Primary Effluent

Samples of unchlorinated and chlorinated primary effluent from the Oak Ridge West Wastewater Treatment Plant (Table 42, Appendix) were collected and processed in the same manner as was the secondary effluent described in the previous section.

Chemical and disinfection data for these samples are given in Table 10. These data for primary effluent do not show the same trends for TKN as determined for secondary effluent (see Table 9). The decrease of TOC on ozonation and uv irradiation is approximately the same for the secondary and primary effluents, but the decrease for chlorinated effluents is less for the primary than with the secondary. The four samples were concentrated approximately 700- to 800-fold and analyzed by HPLC. The chromatograms reveal the varying effects each disinfectant had on the sample. Figure 14 shows significant reduction of components upon ozonation, while chlorination shows less significant reduction of the same components as shown in Figure 19. Figure 20 shows no change in the composition of the sample after uv irradiation. The high oxidation potential of ozone is clearly evident in the destruction of most of the chromatographic peaks. Chlorine being a weaker oxidant destroyed less organics than did ozone.

TABLE 9. COMPARATIVE DISINFECTION OF A FILTERED SECONDARY WASTEWATER EFFLUENT SAMPLE: CHEMICAL AND DISINFECTION DATA

Disinfection treatment	Treatment dosage or residual	Bacteria count (No./mL)[a]	pH	Concentration (mg/L)			
				COD	BOD_5	TKN	TOC
Control	None	277,000	7.3	45	17	3.8	11.5
Ozonated	8 mg O_3/L effluent	500	7.5	40	7	8.2	7.0
Ultraviolet	21,200 μW·s/cm^2	100	7.4	39	<5	7.9	9.6
Chlorinated	Residual chlorine, 0.5 mg/L	2,200	7.3	44	5	9.2	8.9

[a] Standard plate-count method.[32]

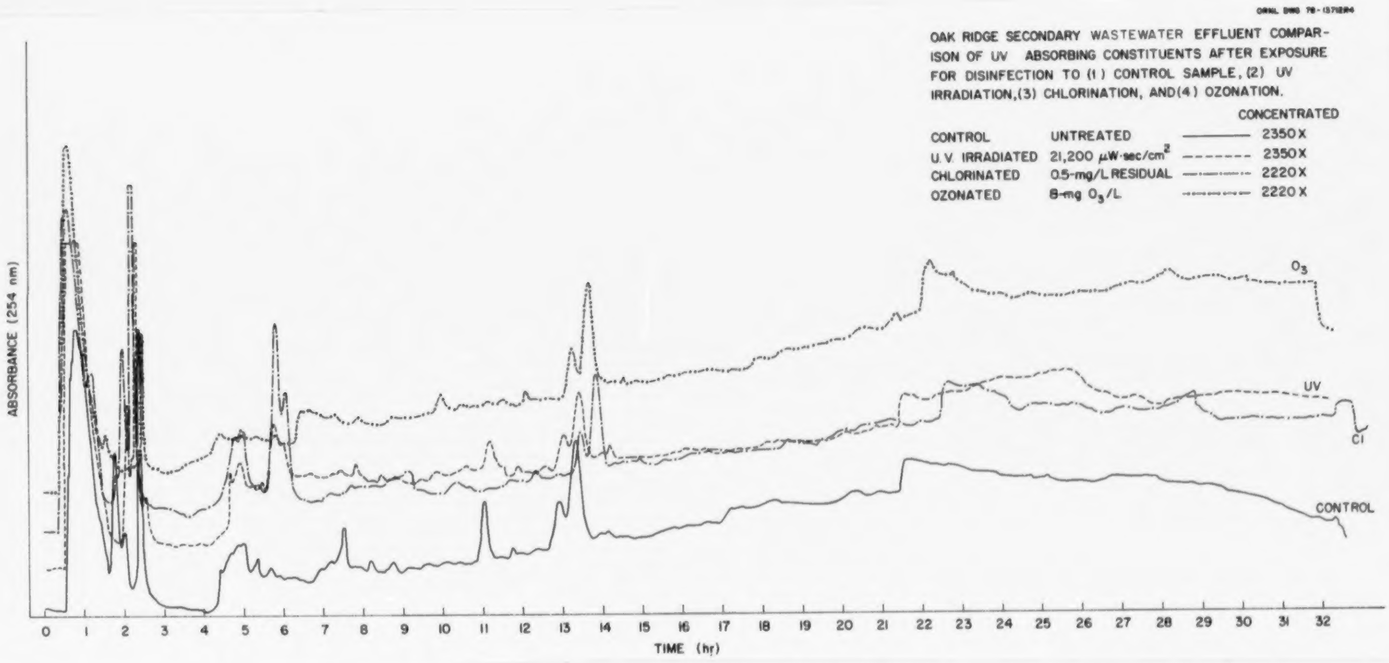

Figure 17. Comparison of chromatograms of uv-absorbing constituents in secondary effluents disinfected with chlorine, ozone, and ultraviolet light.

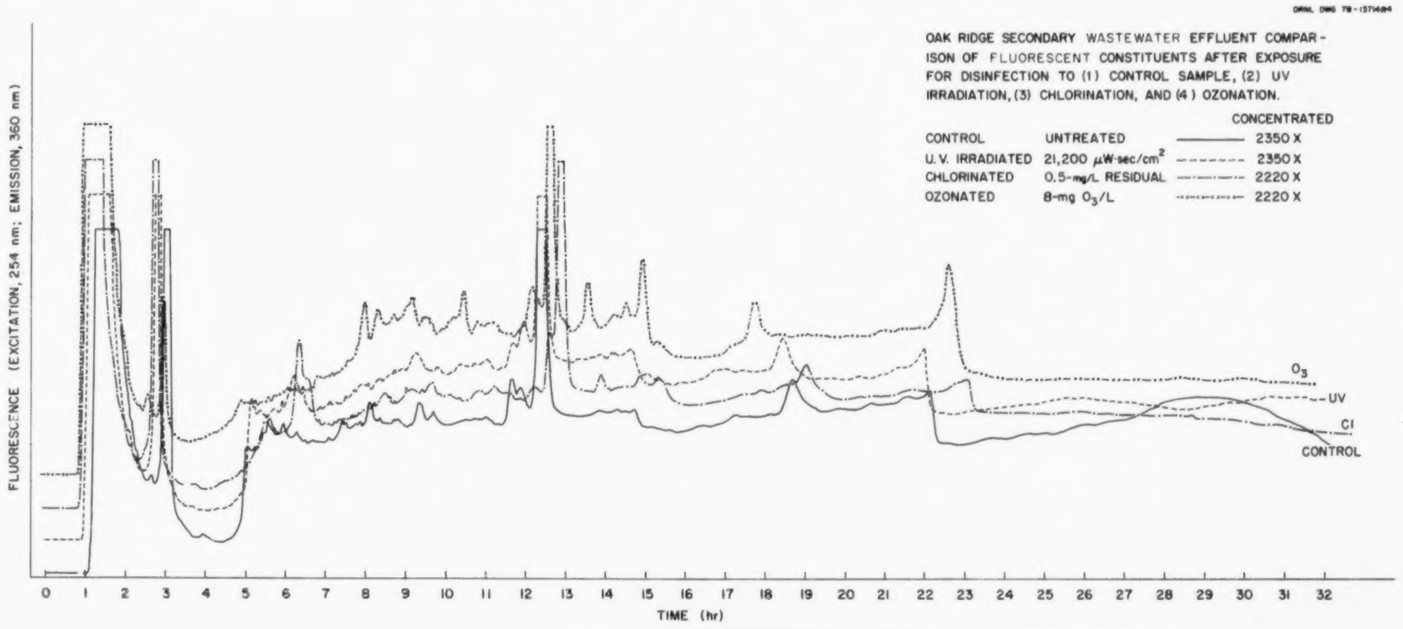

Figure 18. Comparison of chromatograms of cerate-oxidizable constituents in secondary effluents disinfected with chlorine, ozone, and ultraviolet light.

TABLE 10. COMPARATIVE DISINFECTION OF FILTERED PRIMARY WASTEWATER EFFLUENT SAMPLE: CHEMICAL AND DISINFECTION DATA

Sample	pH	Concentration (mg/L) BOD$_5$	COD	TOC	TKN	Total coliform[a] (No./100 mL)
Control	7.6	14	28	20.1	9.2	185,000
Chlorinated (Cl residual, 0.9 mg/L)	7.7	19	42	20.7	8.6	1
Ozonated (6.9 mg ozone/L)	7.9	12	32	17.6	8.4	15
Ultraviolet irradiated (27,500 μW·s/cm^2)	7.6	18	42	17.9	8.8	1

[a] Membrane-filter method.[30]

This relative lack of chemical effect of uv irradiation at levels that disinfect the wastewater may be highly significant because this indicates much less potential for production of toxic reaction products from uv irradiation than from disinfection with chlorine or ozone. However, one field sample collected at Northwest Bergen County Wastewater Treatment Plant that had been disinfected with uv irradiation exhibited some chemical effects due to uv irradiation, as will be discussed later.

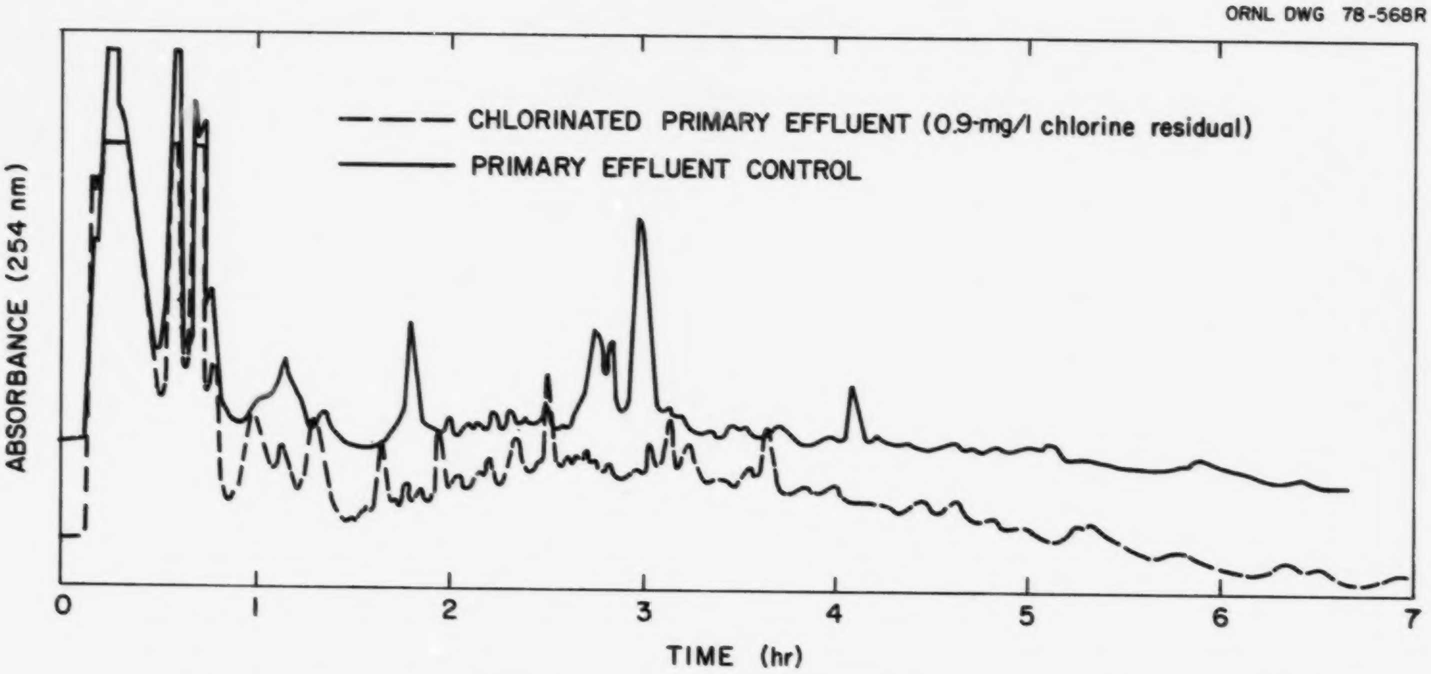

Figure 19. Chromatograms of uv-absorbing constituents in chlorinated primary effluent (chlorine residual, 0.9 mg/L) compared with undisinfected control primary effluent. Samples were collected from the Oak Ridge West Wastewater Treatment Plant; chlorinated sample, 0.77 mL of 800-fold concentrate, unchlorinated sample, 0.77 mL of 700-fold concentrate.

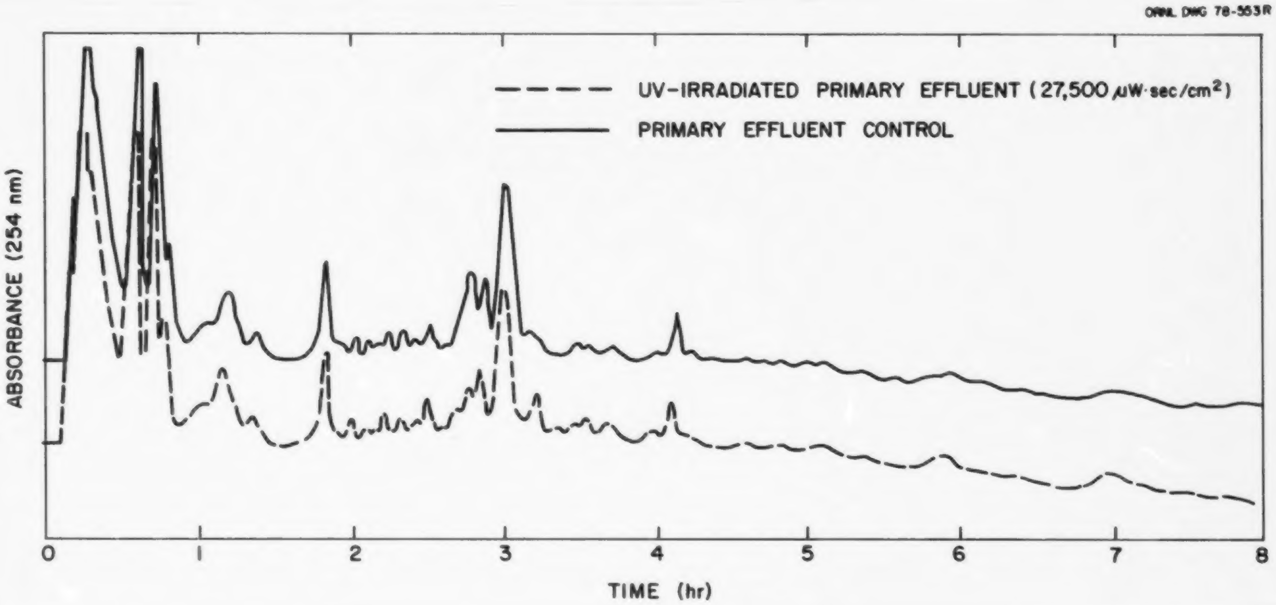

Figure 20. Chromatograms of uv-absorbing constituents in uv-irradiated primary effluent (27,500 $\mu W \cdot s/cm^2$) compared with undisinfected control primary effluent. Samples were collected from the Oak Ridge West Wastewater Treatment Plant and the disinfected sample was uv irradiated in the laboratory; ultraviolet-irradiated sample, 0.77 mL of 660-fold concentrate; unirradiated sample, 0.77 mL of 700-fold concentrate.

SECTION 7

RESULTS OF MUTAGENICITY STUDIES: EFFLUENT CONCENTRATES

The testing of complex mixtures for mutagenic potential presents special problems as compared with testing for pure or individual components. For example, the mutagenicity of some chemical constituents in a mixture may be obscured by the toxicity of the total mixture.[34] Toxicity masks the mutagenicity by killing mutant colonies giving the apparent effect of lower mutagenicity. In the initial stages of development of mutagenicity test procedures for effluent concentrates, it was proposed that the finding of mutagenic activity in the effluent concentrate be the criterion for further characterization of the concentrate by HPLC. However, this criterion was later shown to be invalid because some mutagenic constituents were separated by HPLC from effluent concentrates that tested negative. Conversely, it was also shown that some mutagenic activity found to be present in the crude concentrates was either lost or inactivated by passage through the HPLC column.

Two methods were evaluated for testing effluent concentrates for mutagenic activity: bacterial mutagenicity test systems and unscheduled DNA synthesis.

BACTERIAL MUTAGENICITY TESTING

Preliminary Experiments

The most sensitive or useful bacterial tester strains for nonvolatile organic materials cannot be determined from negative results. Therefore, Oak Ridge primary and secondary effluent concentrates were tested extensively using a variety of bacterial tester strains in an effort to optimize the conditions for the detection of mutagens among the nonvolatile organics of wastewater effluents.

In the preliminary experiments with effluent concentrates, positive results were obtained with the Salmonella typhimurium strain TA-1535. Strain TA-100, which was derived from TA-1535 and is very much more sensitive to many mutagens, was only weakly affected, if at all, by concentrates which were highly mutagenic to TA-1535. Both strains detect base pair substitution mutations. It is interesting to note that TA-100 is more sensitive than TA-1535 for most mutagens affecting these strains, but there are classes of compounds which respond more strongly to TA-1535, for example, some small halogen-containing compounds (personal communication, T. K. Rao, ORNL). The other Ames tester strains, TA-1538 and TA-98, that detect primarily frame-shift mutagens showed no response in preliminary experiments but were used in many subsequent experiments because they were useful in detecting this class of mutagens. The *E. coli* strain WP2, which like TA-1535 should detect base pair substitution mutations, also showed no response in preliminary experiments. Strain WP2 was used in some subsequent experiments because it had previously given results similar to those obtained with TA-1535 for other kinds of samples. The *Salmonella* strain SV3 was used in several experiments but, because of negative results, it was not used extensively.

The 9000 g supernate of the homogenate of the livers from Aroclor-induced rats (S-9) was used in many early experiments to test for possible metabolic activation of indirect mutagens. In these tests S-9 either had no effect or the mutant recovery was reduced. This indicates that the mutagens involved are primarily direct acting and that S-9 may interfere with the test by providing alternative targets for the active electrophiles. Consequently, the use of S-9 was discontinued in later experiments.

Testing of Field Samples

The concentrates of field samples from the various plants included in this research program were tested as completely as possible with current technology. Table 11 summarizes results on effluent concentrates from nine wastewater treatment plants. Most of the tests showed negative results. No convincing evidence of mutagenicity was obtained for either chlorinated or unchlorinated samples from the Oak Ridge East End Wastewater Treatment Plant (ORSE) using a variety of tester strains (Figure 21). This plant primarily treats residential wastewater and carries out secondary treatment. In contrast, clear positive results were obtained for concentrates of chlorinated samples from the Oak Ridge West End Wastewater Treatment Plant (ORPE) in strain TA-1535. There was no positive effect for these samples in WP2 and only a slight effect in TA-100. The positive effect of these samples is substantial, giving over 200 times the control values in the TA-1535 tests (Figure 22); the test was repeated several times for samples taken at different times of the year with similar results. The positive mutagenic effects in samples from ORPE were dependent on chlorination, and unchlorinated samples showed no mutagenicity. The ORPE is the only primary treatment plant included in this study. With TA-1535, concentrates of primary effluents which were treated with ozone or uv irradiation were only very weakly mutagenic, if at all. Based on the criteria used in this study, the tests on ozonated and uv-irradiated primary effluents are reported as negative tests.

A weak positive effect was seen with or without chlorination in concentrates of effluents from the Lyons Bend Wastewater Treatment

TABLE 11. RESULTS OF MUTAGENICITY TESTS ON CONCENTRATES OF EFFLUENTS FROM WASTEWATER TREATMENT PLANTS

Sample source[a]	Disinfection treatment	Treatment dose[b]	Sample date[c]	Concentration factor	Tester strain	Mutagenic activity[d]
ORSE	None		2/7/77	1000	WP2	-
ORSE	None		2/7/77	2000	WP2	-
ORSE	Chlorine	0.6 mg/L	8/2/77	3100	WP2	-
ORSE	None		9/27/77	1000	WP2	-
ORSE	Chlorine	0.5 mg/L	9/27/77	1000	WP2	-
ORSE	None		10/5/77	2350	TA-1535 TA-100	- -
ORSE	Chlorine	0.8 mg/L	10/5/77	2200	TA-1535 TA-100	- -
ORSE	Ozone	8.0 mg/L	10/5/77	2200	WP2 TA-1535	- -
ORSE	UV	21,200 $\mu W \cdot s/cm^2$	10/5/77	2350	WP2 TA-1535	- -
ORSE	Chlorine	0.8 mg/L	1/31/78	2000	WP2	-
ORSE	None		2/8/78	1000	TA-1535	-
ORSE	Chlorine	0.6 mg/L	2/8/78	1000	TA-1535	-
ORPE	None		9/27/77	1000	WP2 TA-1535	- -
ORPE	Chlorine	1.1 mg/L	9/27/77	1000	WP2 TA-1535	- +
ORPE	None		12/12/77	2000	TA-1535	-
ORPE	Chlorine	0.9 mg/L	12/12/77	2000	WP2 TA-1535 TA-100	- + ±
ORPE	Ozone	6.9 mg/L	12/12/77	2000	WP2 TA-1535 TA-100	- - -
ORPE	UV	27,500 $\mu W \cdot s/cm^2$	12/12/77	2000	TA-1535	-
ORPE	None		5/19/78	2000	TA-1535	-
ORPE	Chlorine	0.8 mg/L	5/19/78	2000	TA-1535	+
LBSE	None		5/3/78	2000	WP2 TA-1535 TA-100	- ± -
LBSE	Chlorine	0.9 mg/L	5/3/78	1700	WP2 TA-1535 TA-100	- ± -

TABLE 11. (continued)

Sample source[a]	Disinfection treatment	Treatment dose[b]	Sample date[c]	Concentration factor	Tester strain	Mutagenic activity[d]
MBSE	None		9/27/78	1000	TA-1535 TA-1537	+ -
MBSE	Chlorine	<0.2 mg/L	9/27/78	1000	TA-1535 TA-1537	± -
MBSE	None		7/17/79	2000	TA-1535 TA-1538	± -
MBSE	Chlorine	<0.2 mg/L	7/17/79	2000	TA-1535 TA-1538	+ -
MMSE	None		3/12/79	2000	TA-1535 TA-1538	+ +
MMSE	Chlorine	2.0 mg/L (Total) 1.5 mg/L (Free chlorine)	3/12/79	2000	TA-1535 TA-1538	- -
MMSE	Ozone	6.1 mg/L	3/12/79	2000	TA-1535 TA-1538	- -
EPSE	None		10/19/77	1000	WP2 TA-1535 TA-100	- - -
EPSE	Ozone	~6 mg/L	10/19/77	1000	WP2 TA-1535 TA-100	- - -
EPSE	None		7/9/78	2000	TA-1535	-
EPSE	Ozone	~6 mg/L	7/9/78	2000	TA-1535	-
EPSE	None		8/6/79	2000	TA-1535	-
EPSE	Ozone	~6 mg/L	8/6/79	2000	TA-1535	-
CSE	None		9/12/77	1500	WP2 TA-1535 TA-100	- - -
CSE	Ozone	8.4 mg/L	9/12/77	1600	WP2 TA-1535 TA-100	- - -
CSE	None		5/24/78	2330	TA-1535 TA-100	- -
CSE	Ozone	8.2 mg/L	5/24/78	2150	TA-1535 TA-100	- -

TABLE 11. (continued)

Sample source[a]	Disinfection treatment	Treatment dose[b]	Sample date[c]	Concentration factor	Tester strain	Mutagenic activity[d]
MCSE	None		6/15/78	2000	TA-1535	-
					TA-100	-
					TA-1538	-
					TA-98	-
MCSE	Ozone	~6 mg/L	6/15/78	2000	TA-1535	-
					TA-100	-
					TA-1538	-
					TA-98	-
MCSE	None		10/5/78	2000	TA-1535	-
					TA-1538	-
MCSE	Ozone	~6 mg/L	10/5/78	2000	TA-1535	-
					TA-1538	±
NBCSE	None		4/21/78	3330	WP2	-
					TA-1535	-
					TA-100	-
NBCSE	UV	146,000 $\mu W \cdot s/cm^2$	4/21/78	2700	WP2	-
					TA-1535	-
					TA-100	-
NBCSE	None		8/28/78	1000	TA-1535	-
					TA-1538	-
NBCSE	UV	118,000 $\mu W \cdot s/cm^2$	8/28/78	1000	TA-1535	±
					TA-1538	±
NBCSE	Chlorine	~1 mg/L	8/28/78	1000	TA-1535	±
					TA-1538	-

[a] See Table 1.

[b] For chlorine, the value given is residual chlorine.

[c] See the appendix for effluent data provided by the treatment plant operator.

[d] Tests that showed less than two times the activity of contemporary negative controls are presented as "negative" (-), those which showed from two to five times the activity of the negative controls are called "weakly positive" (±), and those which showed more than five times the activity of negative controls are considered "positive" (+).

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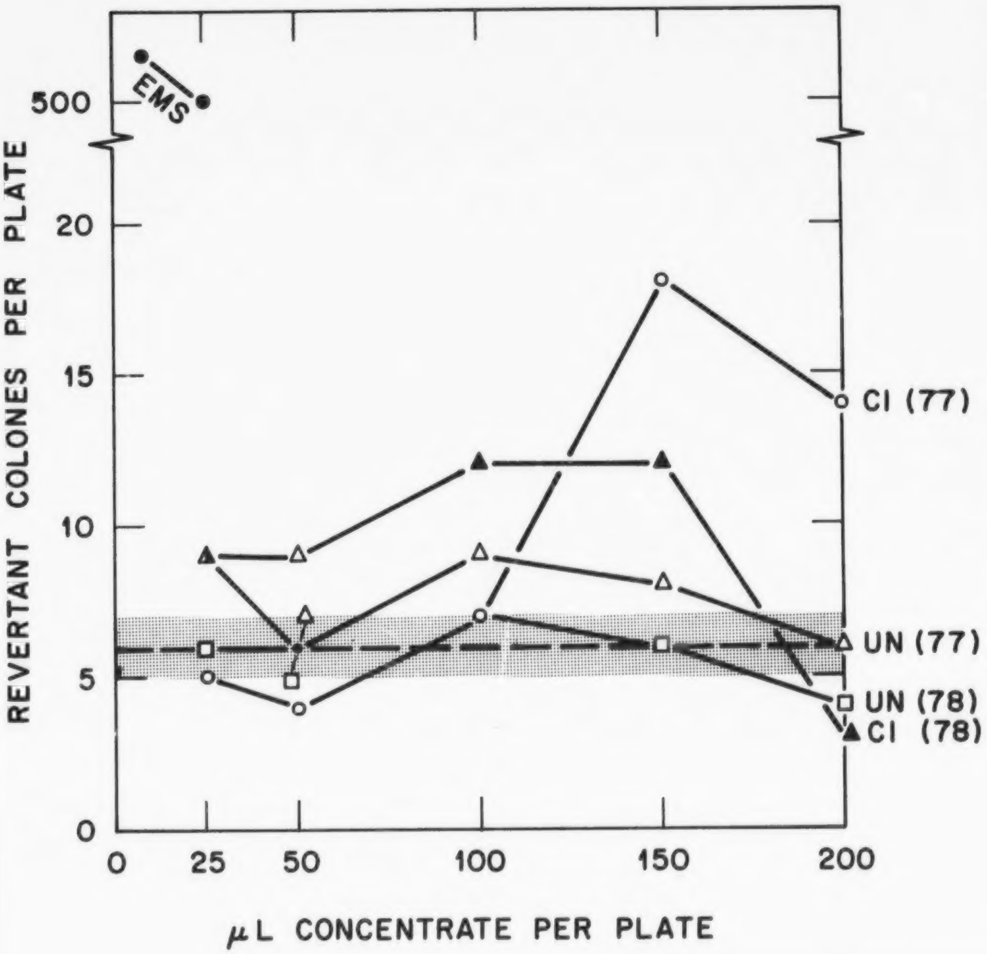

Figure 21. Mutagenic activity tests of ORSE secondary effluent concentrates using Salmonella typhimurium strain TA-1535. Symbols: Cl = chlorinated; UN = not chlorinated; (77) = collection year. The heavy horizontal line represents the negative control. The lines above and below the heavy line represent plus and minus one standard deviation (S.D.).

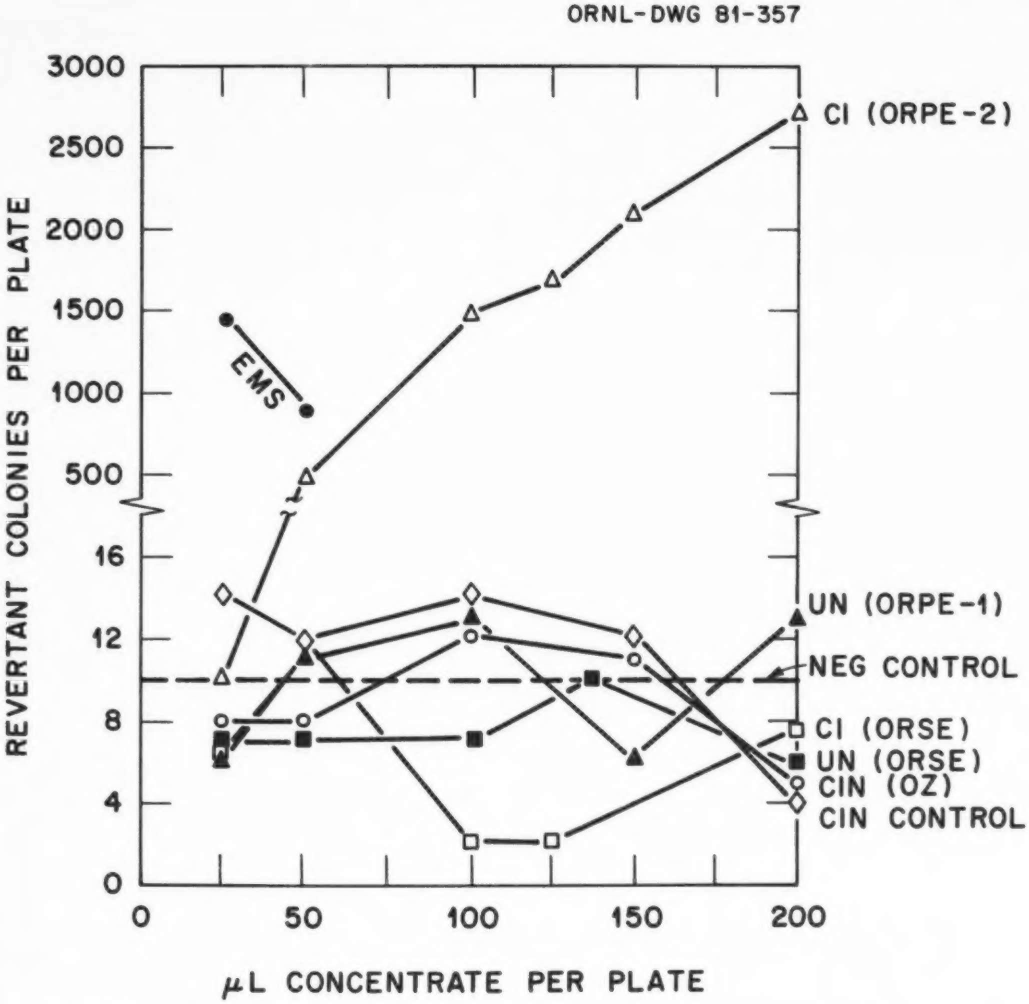

Figure 22. Mutagenic activity tests of CSE effluent concentrates using *Salmonella typhimurium* strain TA-1535. For comparison, results for CSE ozonated and control effluents (9/12/77) are presented as CIN(OZ) and CIN control.

Plant in Knoxville, Tennessee (Figure 23). This plant serves a primarily residential area and does secondary treatment. The effect was seen only with TA-1535. A weak positive effect in strain TA-1535 was also seen in unchlorinated concentrates from the Moccasin Bend Wastewater Treatment Plant in Chattanooga, Tennessee. Chlorination increased this effect in samples collected in the summer (July 1979) but decreased the effect in samples taken the preceding fall (October 1978) (Figure 24). No effects were seen with or without chlorination in concentrates from this plant tested with TA-1538. In concentrates from the Marlborough, Massachusetts, wastewater treatment plant, there was a positive mutagenic effect in undisinfected concentrates. This effect was eliminated both by chlorination and by ozonation of the nitrified effluent (Figure 25). No mutagenic effect was detected in the Northwest Bergen County samples collected in the spring (April 1978). However, although the concentrate of the undisinfected Northwest Bergen County effluent collected in the summer (August 1978) had no mutagenic activity, a weak positive effect was found in concentrates of both the uv-irradiated and the chlorinated effluents (Figure 26). All effluent concentrates for Meander Creek Wastewater Treatment Plant tested negative except for a weak positive effect in the concentrate of the ozonated effluent collected on 10/5/78. The tests in TA-1535 for Meander Creek are shown in Figure 27.

The concentrates from other plants that were tested (CSE and EPSE) gave only negative results in the strains used (Figures 28 and 29). This does not mean that there are no mutagenic components in these treated or untreated effluents, but only that with the strains and procedures used such components, if present, may not have been detected.

Preparation of Test Standards and Characterization of Mutagenic Activity in Effluent Concentrates

Effluent concentrate standards with mutagenic activity and without mutagenic activity were prepared for controls in bacterial mutational tests. With this type of positive and negative control, toxicity effects due to high salt concentrations were more closely duplicated, thus giving a more accurate estimate of true mutagenicity of the effluent concentrate being tested. Therefore, milliliter volumes of concentrates of both unchlorinated and chlorinated primary effluent from the Oak Ridge West Wastewater Treatment Plant (collected 5/19/78, Table 42, Appendix) were prepared for this purpose. A 60-L sample of unchlorinated primary effluent was concentrated 2000-fold. No mutagenic activity was detected for this concentrate (Figure 30). Therefore, it was used as a negative control in subsequent mutagenic tests that required a negative control essentially corresponding in chemical composition to that of the effluent concentrate being tested. Similarly, a 20-L sample of chlorinated primary effluent (residual chlorine, 1.5 mg/L) was concentrated 2000-fold for use as a positive control, i.e., an effluent concentrate with known mutagenic

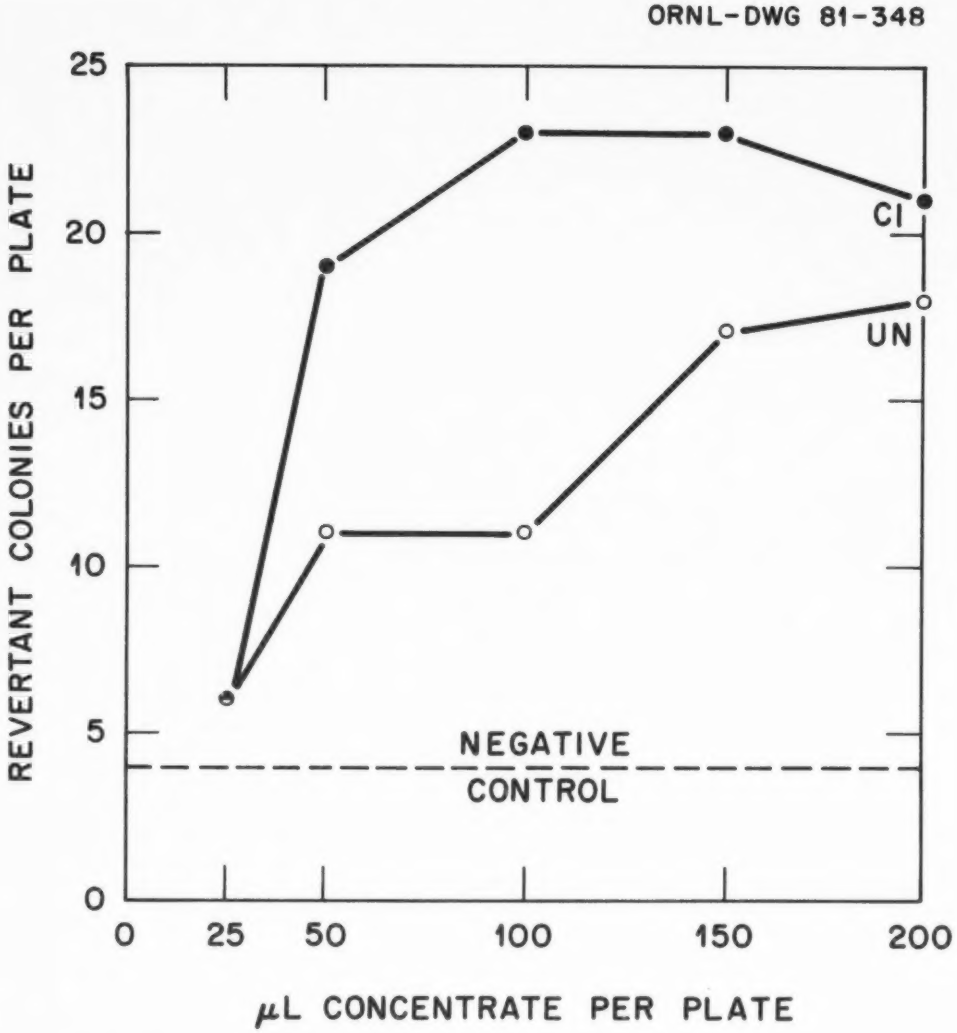

Figure 23. Mutagenic activity tests of LBSE secondary (5/3/78) effluent concentrates using Salmonella typhimurium strain TA-1535. Symbols: Cl = chlorinated; UN = not disinfected.

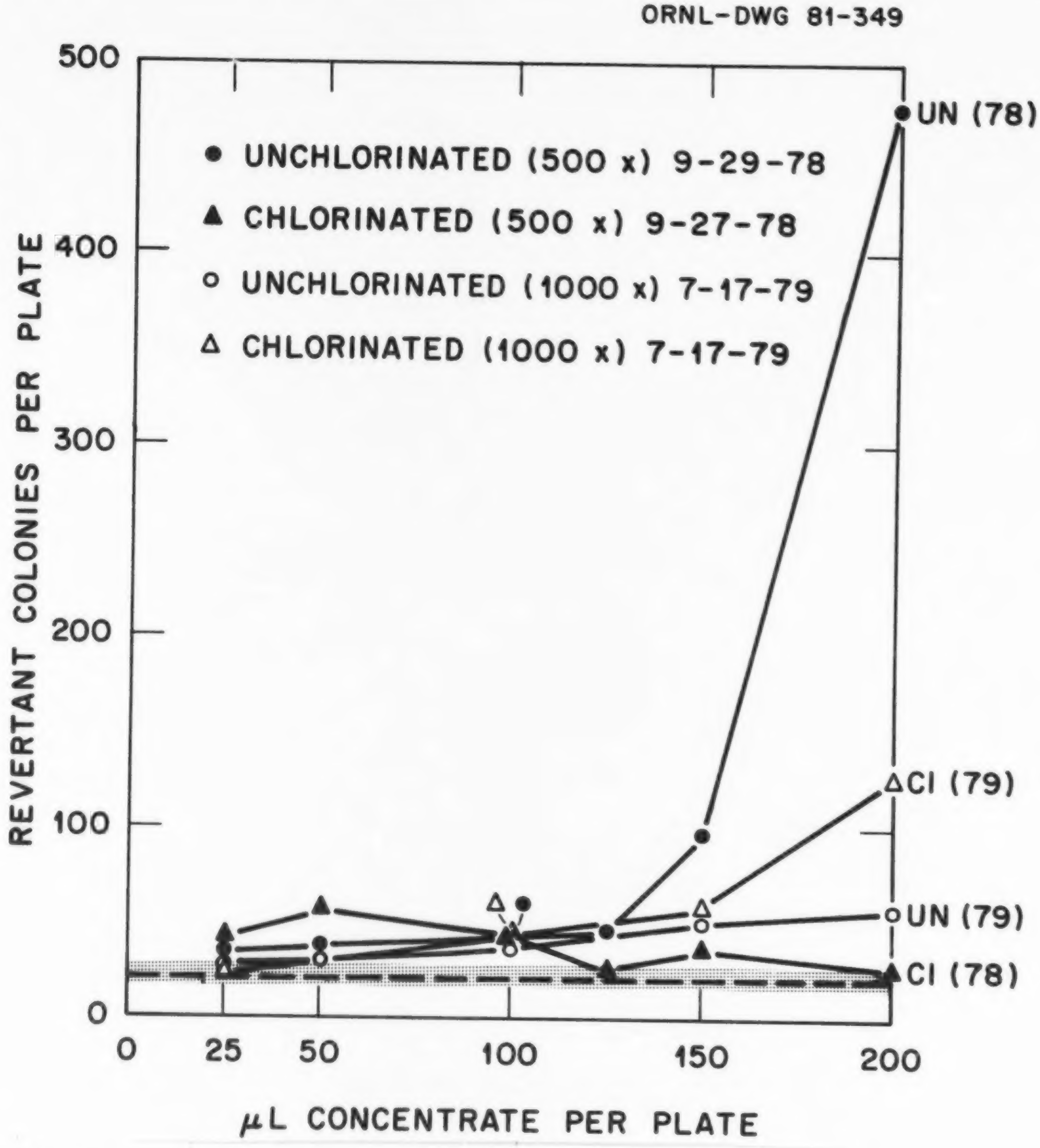

Figure 24. Mutagenic activity tests of MBSE effluent concentrates using Salmonella typhimurium strain TA-1535. Symbols: Cl = chlorinated; UN = not disinfected; (77) = collection year; (500 x) = concentration factor. The heavy horizontal line represents the negative control. The lines above and below the heavy line represent plus and minus one standard deviation (S.D.).

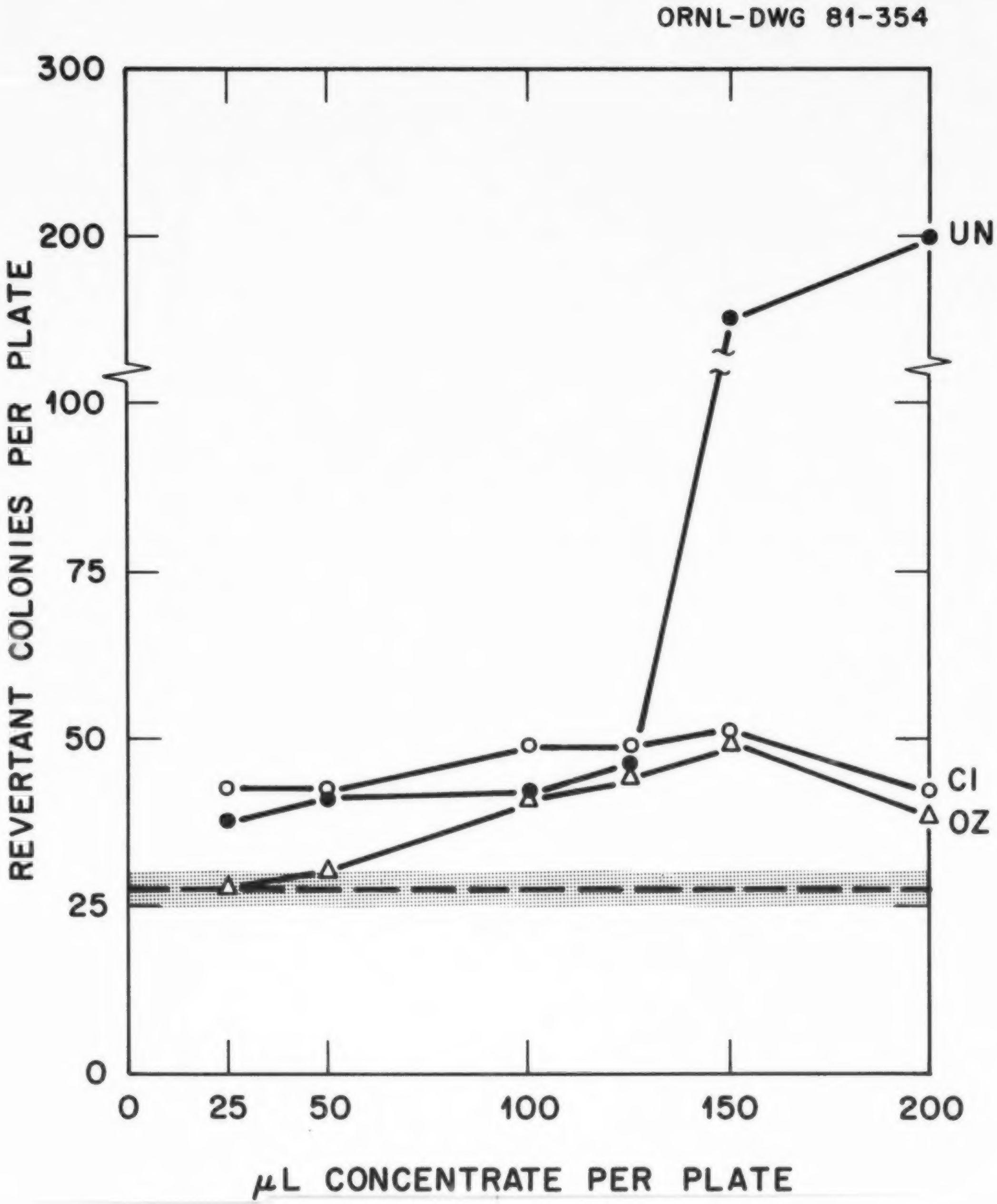

Figure 25. Mutagenic activity tests of MMSE effluent (3/12/79) concentrates using Salmonella typhimurium strain TA-1535. Symbols: OZ = ozonated; Cl = chlorinated; UN = undisinfected. The heavy horizontal line represents the negative control. The lines above and below the heavy line represent plus and minus one standard deviation (S.D.).

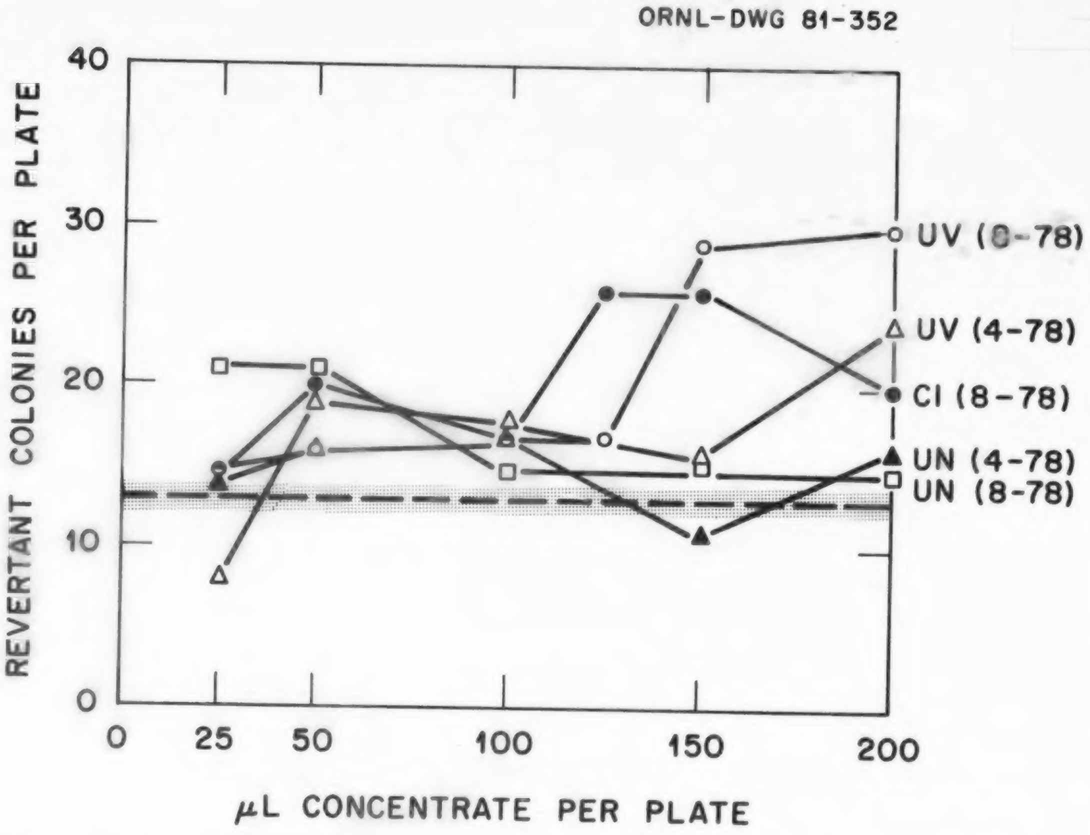

Figure 26. Mutagenic activity tests of NBCSE effluent concentrates using Salmonella typhimurium strain TA-1535. Symbols: UV = uv-irradiated; Cl = chlorinated; UN = not disinfected; (4/78) = collection date. The heavy horizontal line represents the negative control. The lines above and below represent plus and minus one standard deviation (S.D.).

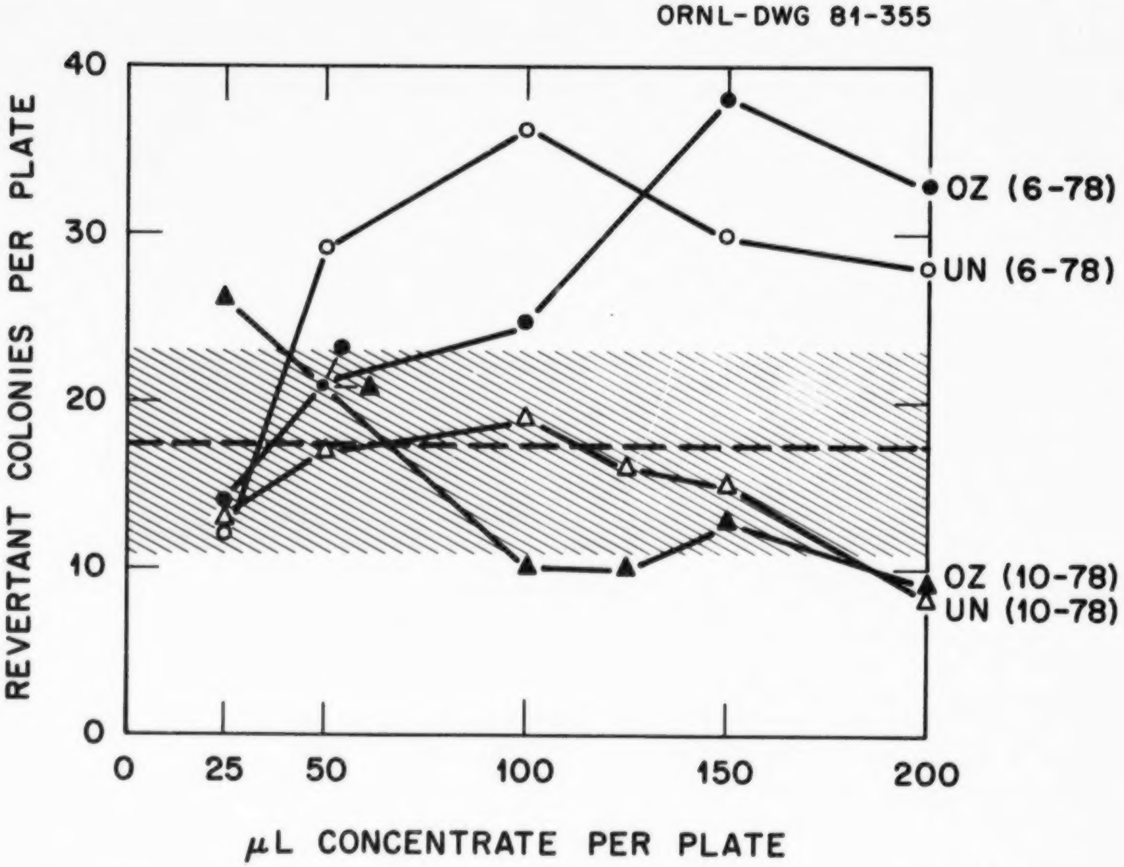

Figure 27. Mutagenic activity tests of MCSE effluent concentrates using Salmonella typhimurium strain TA-1535. Symbols: OZ = ozonated; UN = not disinfected; (6/78) = collection date. The heavy horizontal line represents the negative control. The lines above and below the heavy line represent plus and minus one standard deviation (S.D.).

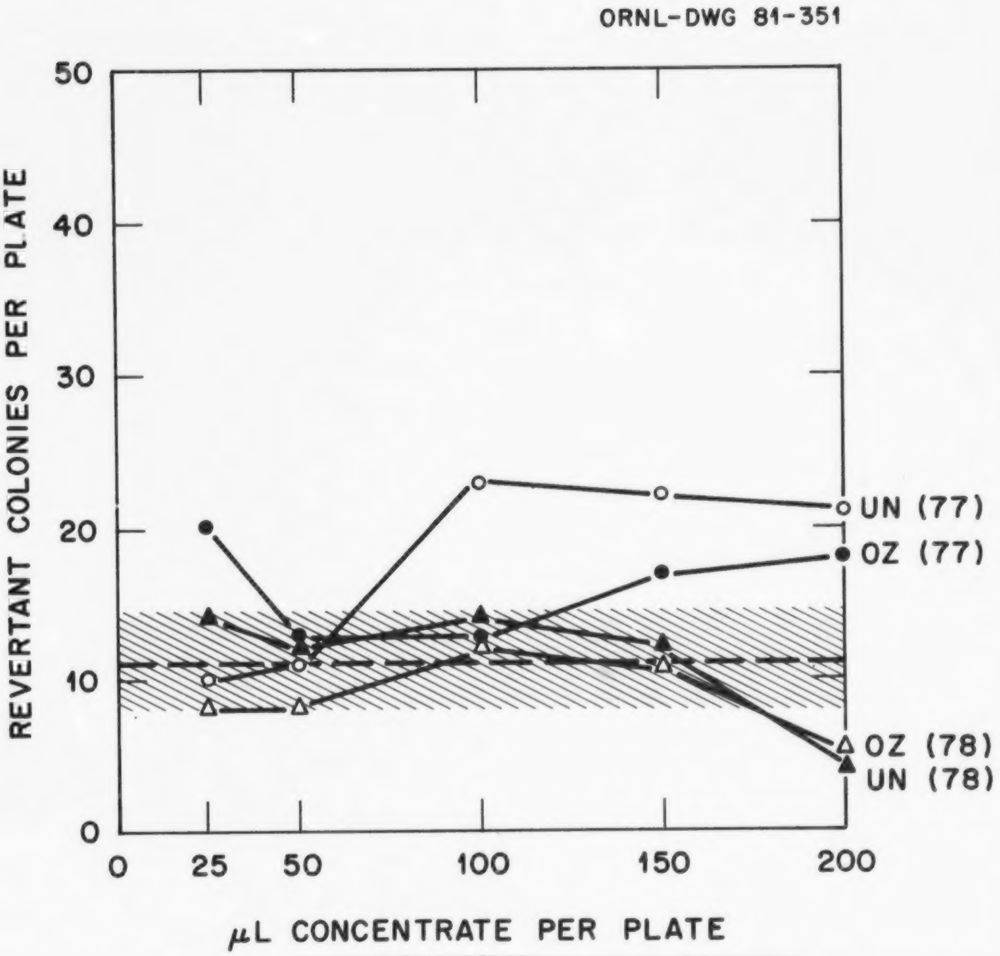

Figure 28. Mutagenic activity tests of CSE effluent concentrates using Salmonella typhimurium strain TA-1535. Symbols: OZ = ozonated; UN = not disinfected; (77) = collection year. The heavy horizontal line represents the negative control. The lines above and below the heavy line represent plus and minus one standard deviation (S.D.).

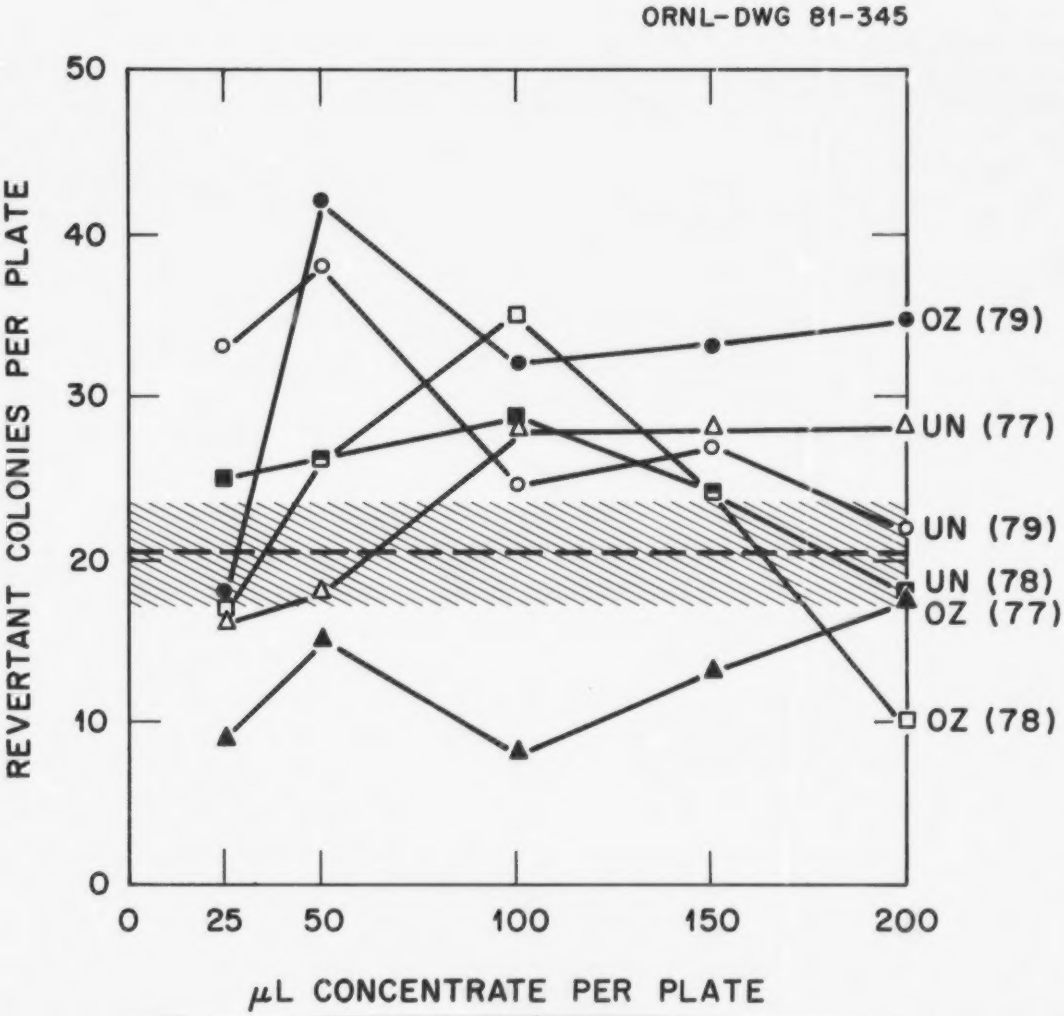

Figure 29. Mutagenic activity tests of EPSE effluent concentrates using Salmonella typhimurium strain TA-1535. Symbols: OZ = ozonated; UN = not disinfected; (77) = collection year. The heavy horizontal line represents the negative control. The lines above and below the heavy line represent plus and minus one standard deviation (S.D.).

activity. To determine whether any of the mutagenic activity of the chlorinated primary effluent was attributable to active chlorine (e.g., organic chloramines) in the concentrates, a 20-L sample of the chlorinated primary was treated with sufficient thiosulfate solution to destroy the residual chlorine and then concentrated 2000-fold. All effluent concentrates were tested for mutagenicity three times each with TA-1535 and once each with SV3. Strain SV3 gave negative results for all samples. The replicates with TA-1535 were always consistent and showed a negative result for the unchlorinated effluent, a strong positive effect for the chlorinated effluents, and an even stronger positive effect for the thiosulfate-treated samples (see Figure 30). The repeatability of these experiments is impressive. It confirmed previous findings of the dependence of the mutagenic effect in the concentrate of Oak Ridge primary effluent on chlorination. The experiment was intended to determine whether the mutagenic effect was due to available chlorine in the effluent concentrates, and the results show that it was not. In addition, whatever is mutagenic in these wastewaters is resistant to thiosulfate and thus is different from the highly reactive alkylating agents [e.g., ethylmethanesulfonate (EMS)] used as positive controls in the bacterial mutagenic activity tests.

Evaluation of Macroreticular Resin for Concentration of Mutagenic Activity

In the later phases of this research program, the macroreticular resins XAD-2 and XAD-7 were evaluated in two tests as a possible alternate method for concentrating organic and mutagenic constituents from wastewater effluents.

Two 20-L samples of chlorinated primary effluent from the Oak Ridge West Wastewater Treatment Plant (collected 11/29/78, Table 42, Appendix) were passed through tandem columns containing XAD resins at approximately 2 L/hr. The first sample was passed through XAD-7 followed by XAD-2 (Run X-72). The second sample was passed through XAD-2 followed by XAD-7 (Run X-27). Each column was 1.2 by 25 cm. Each of the four columns was eluted with 250 mL of acetone, and the resulting acetone solutions were evaporated to dryness and weighed. The residue from all four columns was a viscous, dark brown oil. This was dissolved in dimethylsulfoxide at 40 mg/mL, and the resulting liquid was tested for mutagenicity with TA-1535 and TA-1538. The amounts of organics recovered and their mutagenic activity are given in Table 12.

The relative lack of mutagenic effect in the X-72 series may be due to toxicity of these samples. This technique shows promise for the concentration of mutagenic components from wastewaters, and XAD-2 appears to be more effective than XAD-7 for isolation of such constituents. However, this method may not efficiently concentrate all nonvolatile constituents although it also concentrates some compounds considered relatively volatile.[21]

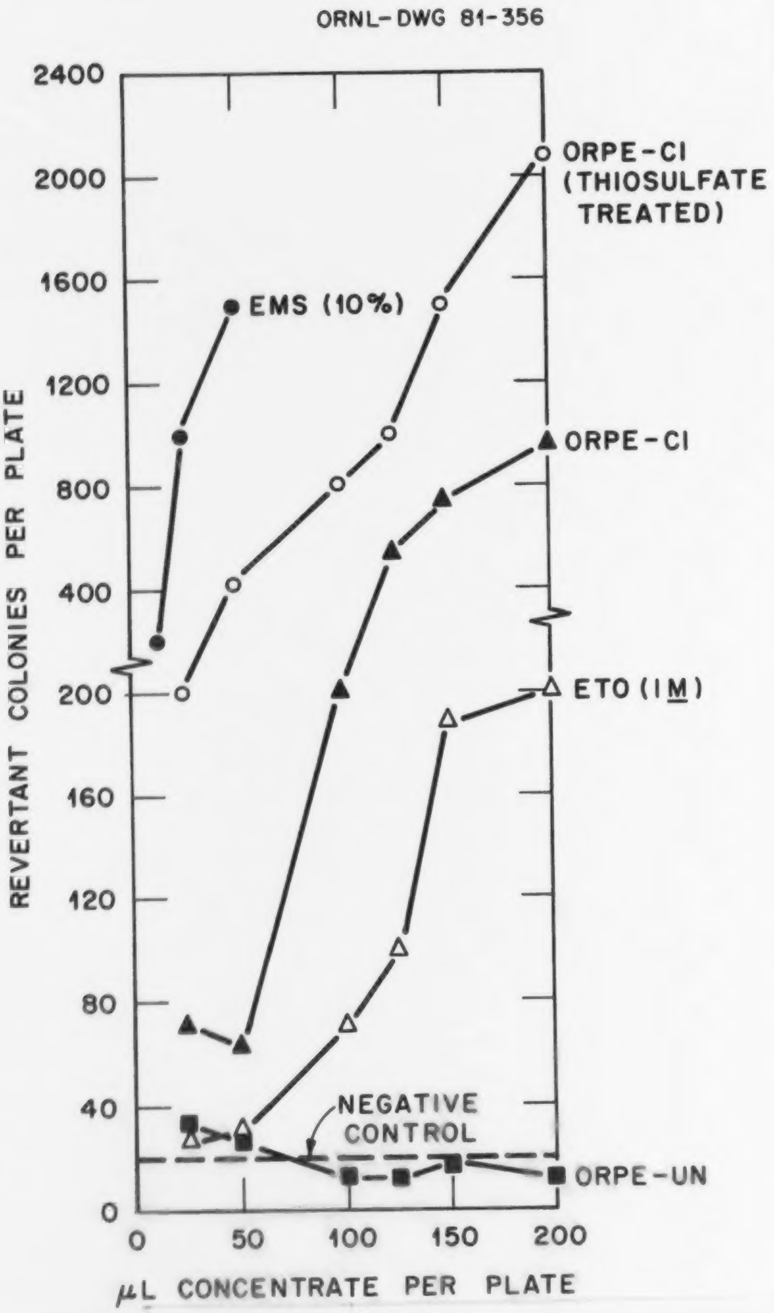

Figure 30. Mutagenic activity tests of concentrates of ORPE undisinfected, chlorinated, and chlorinated (thiosulfate-treated) effluents. Symbols: Cl = chlorinated; UN = undisinfected; EMS = ethylmethanesulfonate in dimethylsulfoxide solution; ETO = ethylene oxide in dimethylsulfoxide solution.

TABLE 12. ORGANIC AND MUTAGENIC CONSTITUENTS ISOLATED FROM CHLORINATED EFFLUENT FROM THE PRIMARY STAGE OF OAK RIDGE WEST WASTEWATER TREATMENT PLANT

Sample	Resin	Organics recovered[a] (mg/L)	Mutagenic activity[b] TA-1535	Mutagenic activity[b] TA-1538
X-72-A[c]	XAD-7	10.5	–	–
X-72-B[d]	XAD-2	7.8	±	±
X-27-A[e]	XAD-2	9.4	+	+
X-27-B[f]	XAD-7	1.4	+	+

[a] Weight organics recoverd per liter effluent.

[b] No mutagenic activity, –; slightly positive mutagenic activity (2 to 5 x negative control), ±; positive mutagenic activity (greater than 5 x negative control), +. Tests were all run with equal concentrations of organic, 40 mg/mL DMSO, except X-27-B sample which was 28 mg/mL DMSO.

[c] Run X-72, first column.

[d] Run X-72, second column.

[e] Run X-27, first column.

[f] Run X-27, second column.

The organic materials separated by adsorption on XAD resin in the X-72 and X-27 runs were compared chromatographically with the nonvolatile organics concentrated by lyophilization from a 20-L aliquot of the same chlorinated primary effluent sample used for the XAD adsorption tests. The HPLC chromatograms of the uv-absorbing and fluorescent constituents separated from the X-72 and X-27 samples and the chromatograms of the concentrate prepared by lyophilization (normal run) are shown in Figures 31 and 32, respectively. Except for small differences at 3 and 5 hr, the uv chromatograms for the X-72, X-27 and normal HPLC runs are very similar. Thus, it appears that many uv-absorbing constituents are concentrated to approximately the same extent by XAD-2 and -7 resins as by vacuum concentration. The response of the fluorescence detector is considerably less for components of runs X-72 and X-27 (peaks are magnified 2.5 x) than for the normal run; two large peaks at 2 and 6 hr in the normal chromatogram are not present in the X-72 and X-27 chromatograms. Some fluorescent constituents are apparently adsorbed (concentrated) less efficiently by XAD-2 and -7 resins than by vacuum concentration. Glaze and coworkers have reported that natural organics after chlorination do not adsorb efficiently on XAD resins.[22] Concentration using XAD-2 followed by XAD-7 is chemically equivalent to using XAD-7 followed by XAD-2.

UNSCHEDULED DNA SYNTHESIS TEST

A study of unscheduled DNA synthesis in mice was carried out using chlorinated secondary effluent from the Oak Ridge East Wastewater Treatment Plant (collected 1/31/78, Table 43, Appendix). A 100-L sample of effluent was concentrated 1000-fold by lyophlization. One set of mice (negative control) was injected with 0.5 mL each isotonic saline solution at the time of tritiated thymidine ($[^3\text{H}]$dTHd) administration. Each mouse of a second set (positive control) was injected with 0.5 mL of saline solution containing 80 mg methylmethanesulfonate (MMS) per kilogram of body weight at the time of $[^3\text{H}]$dThd administration. Each mouse of a third set (Test 1) was injected with 2 μL of effluent concentrate per gram of body weight at the time of $[^3\text{H}]$dThd administration. A fourth set (Test 2) was treated the same as the third set except the effluent concentrate was injected 30 min prior to $[^3\text{H}]$dThd administration.

The unscheduled DNA synthesis studies with the 1000-fold concentrate of chlorinated secondary effluent were negative (Table 13). However, they did show substantial toxicity to mammals from the nonvolatile organics in the chlorinated secondary effluents. The concentrate of chlorinated effluent was negative in bacterial mutagenicity tests. In order to more fully reveal the effects of chlorination on constituents in wastewater, further unscheduled DNA synthesis tests are necessary with samples that were positive in bacterial tests, e.g., chlorinated primary effluents.

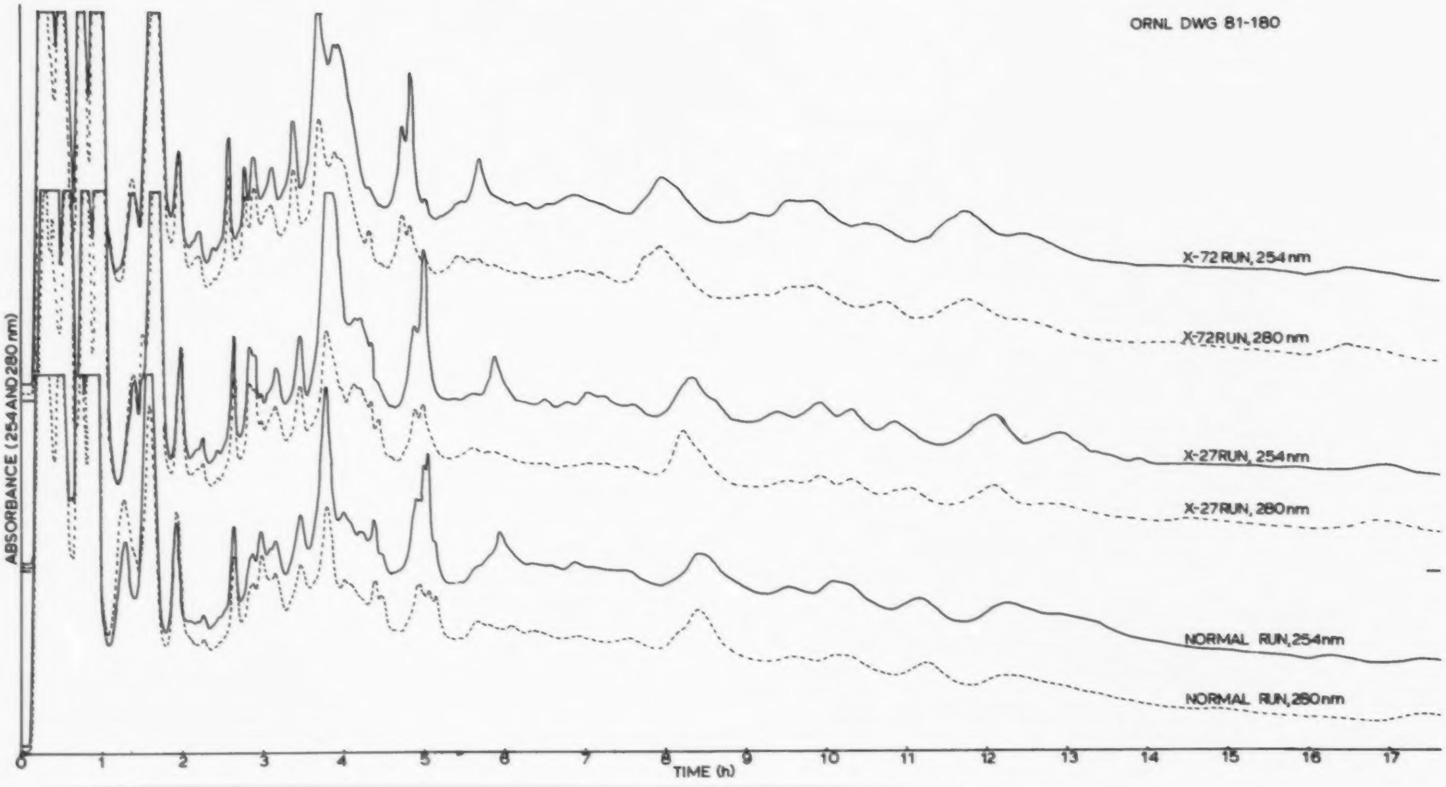

Figure 31. HPLC chromatograms of uv-absorbing constituents concentrated by adsorption on XAD resins (X-72 and X-27 runs) as compared with uv-absorbing constituents concentrated by lyophilization (normal run). Each HPLC run represents chromatography of an equivalent amount of wastewater effluent.

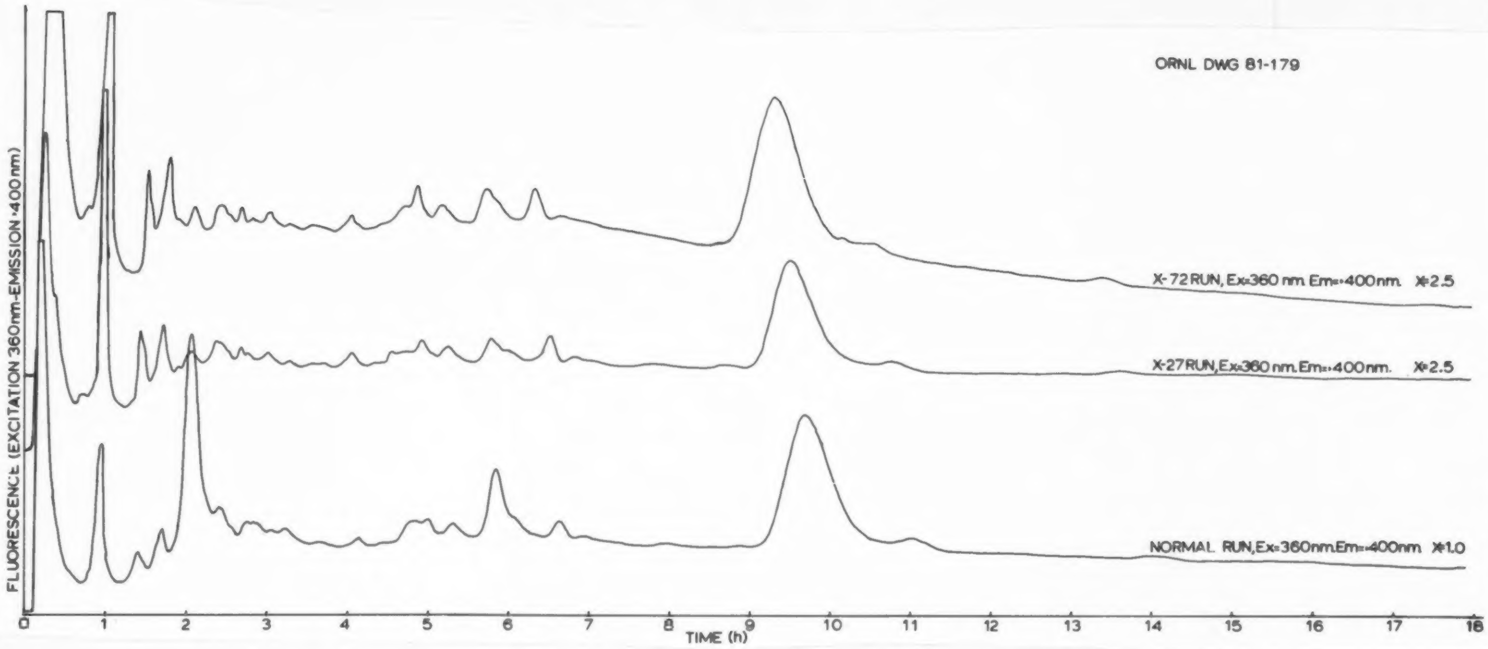

Figure 32. HPLC chromatograms of fluorescent constituents concentrated by adsorption on XAD resins (X-72 and X-27 runs) as compared with fluorescent constituents concentrated by lyophilization (normal run). Each HPLC run represents chromatography of an equivalent amount of wastewater effluent. However, the attenuation for X-27 and X-72 runs was adjusted 2.5x to magnify peaks.

TABLE 13. EXPERIMENTAL RESULTS FROM UNSCHEDULED DNA SYNTHESIS STUDY USING A 1000-FOLD CONCENTRATE OF UNCHLORINATED SECONDARY EFFLUENT

Test	Treatment	Radioactivity ($[^3H]$dpm/sperm cell)[a]
Negative control	Saline solution (0.5 mL/mouse)	5.1×10^{-6}
Positive control	MMS (80 mg/kg body wt) in saline solution	180×10^{-6}
Test 1	Effluent concentrate (2 μL/g body wt)	2.6×10^{-6}
Test 2	Effluent concentrate (2 μL/g body wt) 30 min before $[^3H]$dThd	5.4×10^{-6}

[a] Tritium concentration in disintegrations per minute per sperm cell.

SECTION 8

FRACTIONATION, CHEMICAL, AND MUTAGENIC ACTIVITY TESTING

A major objective of this research program was to determine chemical effects of the disinfectants (chlorine, ozone, and ultraviolet-light irradiation) on nonvolatile organic constituents in effluents from selected wastewater treatment plants. Therefore, concentrates of effluents from nine wastewater treatment plants were analyzed by HPLC, and separated fractions were collected for further testing. A second objective was to test fractions for mutagenic activity. Because approximately 250 eluate fractions were collected in each HPLC separation, they were pooled into 20 to 30 collective fractions to reduce the number of complex bacterial mutagenicity tests to a more manageable and economical level. Fractions showing positive mutagenic activity were targeted for identification of its individual components.

Preliminary HPLC experiments were conducted with concentrates of Oak Ridge effluents. Subsequently, effluent samples from seven other wastewater treatment plants were examined.

PRELIMINARY EXPERIMENTS

To isolate the mutagenic compounds from the chlorinated Oak Ridge primary effluent concentrate (collected 5/19/78, Table 42, Appendix) previously found highly mutagenic it was chromatographed by HPLC and separated fractions were pooled for testing.

Of the fractions so collected, eight were weakly positive in a mutational assay with TA-1535 (see Table 14 and Figure 33). The total mutagenicity recovered was far less than was present in the original concentrate. This result raised the possibility that some mutagenic species are either lost or inactivated during the HPLC separation procedure.

The eluate fractions separated by HPLC from the concentrate of undisinfected Oak Ridge secondary effluent (collected 5/15/79, Table 43, Appendix) were pooled into 22 fractions. They were tested in TA-1535 and TA-1538, but no mutagenicity was observed in any of the fractions. These results are consistent with previous negative data determined for the concentrates of Oak Ridge secondary effluent.

TABLE 14. MUTAGENIC ACTIVITY OF CHROMATOGRAPHIC FRACTIONS OF CHLORINATED PRIMARY EFFLUENT FROM OAK RIDGE WEST WASTEWATER TREATMENT PLANT (MAY 19, 1978)[a]

	Chromatographic separation no. 1 (Figure 33)			
Sample no.	Fraction no.	Peak (uv) present	Result TA 1535	No. of tests
1	1-7	+	-	1
2	8-10	+	±	2
3	11-15	+	±	2
4	16-20	+	-	1
5	21-23	+	-	1
6	24-28	+	-	1
7	29-32	+	-	1
8	33-37	+	±	2
9	38-43	+	-	1
10	44-47	+	-	1
11	48-51	+	±	2
12	52-58	+	-	1
13	59-62	-	-	1
14	63-68	-	-	1
15	69-73	+	-	1
16	74-77	-	-	1
17	78-80	+	-	1
18	81-88	+	±	2
19	89-94	+	-	1
20	95-107	+	-	1
21	108-114	+	-	1
22	115-124	-	-	1
23	125-133	+	-	1
24	134-145	+	-	1
25	146-155	+	±	2
26	156-170	-	±	2
27	171-185	-	-	1
28	186-200	+	±	2
29	201-220	-	-	1
Total				36

[a] Tests run with appropriate positive and negative controls.

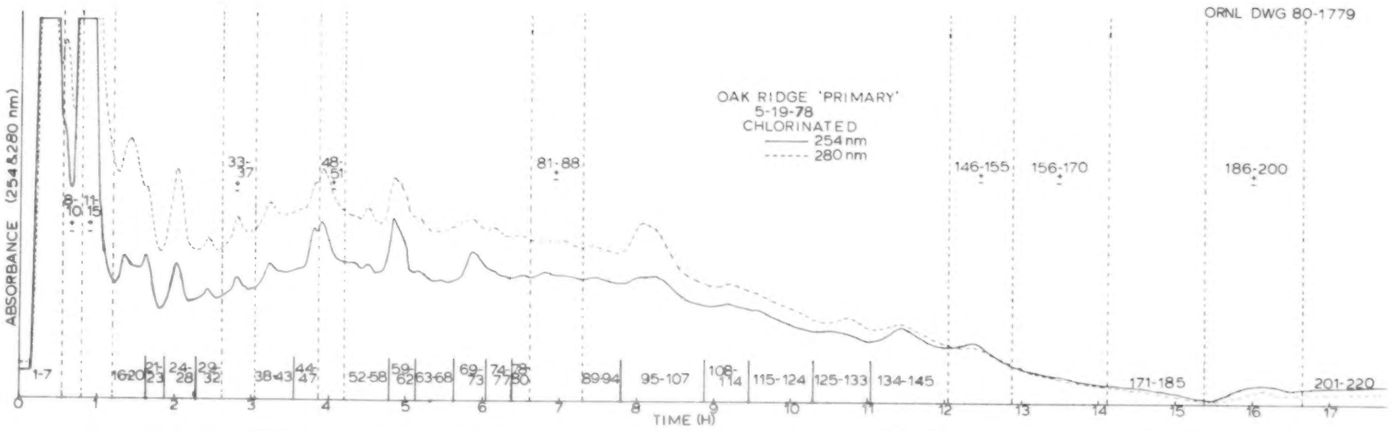

Figure 33. Chromatogram of Oak Ridge chlorinated primary effluent (concentrated 2000-fold) showing mutagenic fractions.

Data for mutagenicity tests with TA-1535 for the HPLC eluate fractions separated from the Oak Ridge samples and for the field samples are summarized in Table 15.

FIELD SAMPLES

Effluent samples were collected at each of the seven wastewater treatment plants selected for this phase of the study which included preparative HPLC and collection of fractions for mutagenic tests and chemical characterization. Table 16 gives a summary of the treatment and characterization efforts for the samples. Effluent data and treatment plant operation parameters as supplied by the treatment plant operators are given in the Appendix. The experimental results, both chemical and mutagenic, are presented in the following sections for each field sample site.

Lyons Bend Fourth Creek Wastewater Treatment Plant (LBSE)

Two sets of samples were collected at the Lyons Bend Fourth Creek Wastewater Treatment Plant, Knoxville, Tennessee, on 5/3/78 and 10/25/78. Each set consisted of unchlorinated (control) and chlorinated effluents. The two sets of samples were concentrated 1000-fold and 2000-fold respectively and examined.

Set 1--

Mutagenicity--The concentrates of the undisinfected and chlorinated (residual chlorine, 0.9 mg/L) effluents (5/3/78) were weakly mutagenic (see Table 11). Figures 34 and 35 show the chromatograms of the uv-absorbing constituents in these samples. Efforts were made to maximize the amount of nonvolatile organics chromatographed, and consequently the columns approached overloading. This effect can be seen by comparing Figures 34 and 35 with Figure 36, which shows analytical-scale chromatograms of the effluent concentrates. Subsequently, the loading for preparative-scale HPLC runs was reduced slightly. Considerable judgment was necessary to maximize organics separated while also maximizing resolution.

A series of 21 pooled chromatographic eluate samples from the undisinfected wastewater concentrate were tested for mutagenic activity using TA-1535 and TA-1538. All were negative in TA-1538. Samples 3 and 14 were slightly positive with TA-1535 (Figure 34 and Table 17).

Thirty chromatographic fractions of the chlorinated wastewater concentrate were tested with TA-1535 and TA-1538. One sample (2) was positive and five others (7, 13, 14, 17, and 23) were slightly positive in TA-1535. Three samples (25, 28, and 30) were slightly positive in TA-1538. These results show that the mutagenicity in

TABLE 15. MUTAGENIC ACTIVITY OF WASTEWATER CHROMATOGRAPHIC FRACTIONS

			Mutagenic activity^c					
			TA-1535			TA-1538		
Sample^a	Sample date^b	Concentration factor	Pos.	Slightly pos.	Neg.	Pos.	Slightly pos.	Neg.
ORSE	5-15-79	1000	0/22	0/22	22/22	0/22	0/22	22/22
ORPE-Cl	5-19-78	2000	0/29	8/29	2/29	No data		
LBSE	5-3-78	2000	0/21	2/21	19/21	0/21	0/21	21/21
LBSE-Cl	5-3-78	2000	1/30	5/30	24/30	0/30	3/30	27/30
MBSE	9-29-78	1000	1/25	6/25	18/25	0/25	2/25	23/25
MBSE-Cl	9-27-78	1000	0/32	2/32	30/32	0/32	7/32	25/32
MBSE	7-17-79	1000	0/31	2/31	29/31	1/31	7/31	23/31
MBSE-Cl	7-17-79	1000	1/22	1/22	20/22	1/22	1/22	20/22
MMSE	3-12-79	2000	0/31	0/31	31/31	0/31	1/31	30/31
MMSE-Oz	3-12-79	2000	4/16	1/16	11/16	4/16	1/16	11/16
MMSE-Cl	3-12-79	2000	2/24	0/24	22/24	2/24	0/29	22/29
MMSE	9-19-79	2000	0/23	6/23	17/23	0/23	5/23	18/23
MMSE-Oz	9-19-79	2000	0/24	1/24	23/24	0/24	1/24	23/24
MMSE-Cl	9-19-79	2000	2/23	2/23	19/23	4/23	4/23	15/23
EPSE	7-9-78	2000	2/33	6/33	25/33	0/33	5/33	28/33
EPSE-Oz	7-9-78	2000	3/38	6/38	28/38	5/38	8/38	25/38
EPSE	8-16-79	2000	0/34	1/34	33/34	0/34	1/34	33/34
EPSE-Oz	8-16-79	2000	1/27	1/27	25/27	1/27	4/27	22/27
CSE	5-24-78	2000	0/24	2/24	22/24	0/24	0/24	24/24
CSE-Oz	5-24-78	2000	0/22	2/22	20/22	0/22	0/22	22/22
MCSE	6-15-78	2000	1/19	0/19	18/19	1/19	0/19	18/19
MCSE-Oz	6-15-78	2000	No data			No data		
MCSE	10-5-78	2000	1/28	5/28	22/28	0/28	1/28	27/28
MCSE-Oz	10-5-78	2000	Data unacceptable					

TABLE 15. (continued)

			Mutagenic activity[c]					
			TA-1535			TA-1538		
Sample[a]	Sample date[b]	Concentration factor	Pos.	Slightly pos.	Neg.	Pos.	Slightly pos.	Neg.
NBCSE	8-28-78	2000	3/18	0/18	15/18	0/18	1/18	17/18
NBCSE-Cl	8-28-78	2000	0/25	0/25	25/25	0/25	0/25	24/25
NBCSE-uv	8-28-78	2000	0/19	0/19	19/19	0/19	0/19	19/19

[a] See Table 1. Symbols: Cl = chlorinated effluent; Oz = ozonated effluent; uv = ultraviolet-light-irradiated effluent.

[b] See Appendix.

[c] Tests that showed less than two times the activity of contemporary negative controls are presented as "negative" (-), those which showed from two to five times the activity of the negative controls are called "weakly positive" (±), and those which showed more than five times the activity of negative controls are considered "positive" (+). Data are presented in ratios (e.g., 24/24) representing the number of positive, slightly positive or negative tests relative to the number of HPLC fractions.

TABLE 16. FIELD SAMPLES: COLLECTION AND PROCESSING DATA

Source and treatment of sample[a]	Set 1			Set 2			Set 3	
	Control	Disinfected		Control	Disinfected		Control	Disinfected
LBSE	5/3/78	5/3/78[b]		10/25/78	10/25/78[b]			
Concentration factor	2860	2670		2000	2000			
uv Spectra, no.	21	30		31	33			
GC, no.	6	9		3	4			
GC/MS, no.	6	9		3	4			
MBSE	9/27/78	9/27/78[b]		7/17/79	7/17/79[b]			
Concentration factor	1000	1000		1000	1000			
uv Spectra, no.	25	32		30	31			
GC, no.	5	4		9	3			
GC/MS, no.	5	4		9	3			
MMSE	3/12/79	3/12/79[c]	3/12/79[b]	9/19/79	9/19/29[c]	9/15/79[b]		
Concentration factor	2000	2000	2000	2000	2000	2000		
uv Spectra, no.	34	20	29	22	24	23		
GC, no.		3		19	21	18		
GC/MS, no.		3		19	21	18		
EPSE	10/19/77	10/19/77[c]		7/8/78	7/8/78[c]		8/6/79	8/6/79[c]
Concentration factor	1560	1600		2000	2000		2000	2000
uv Spectra, no.	40	51		33	38		38	27
GC, no.	22	4		1	2		7	9
GC/MS, no.	22	4		1	2		7	9
CSE	9/12/77	9/12/77[c]		5/24/78	5/24/78[c]			
Concentration factor	1490	1600		2330	2150			
uv Spectra, no.	46	41		24	22			
GC, no.	16	11		22	12			
GC MS, no.	16	11		21	12			

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TABLE 16. (continued)

Source and treatment of sample[a]	Set 1		Set 2			Set 3	
	Control	Disinfected	Control	Disinfected		Control	Disinfected
MCSE	6/15/78	6/15/78[c]	10/5/78	10/5/78[c]			
Concentration factor	2000	2350	2000	2000			
uv Spectra, no.	19	20	28	27			
GC, no.			8				
GC/MS, no.			8				
NBCSE	4/21/78	4/21/78[d]	8/28/78	8/28/78[d]	8/28/78[b]		
Concentration factor	3300	2700	2000	2000	2000		
uv Spectra, no.	26	26	18	15	25		
GC, no.	2	2	7	2			
GC/MS, no.	2	2	7	2			

[a] See Table 1 and Appendix for effluent data provided by the treatment plant operator. All samples were subjected to both analytical-scale and preparative scale HPLC.

[b] Disinfected by chlorine.

[c] Disinfected by ozone.

[d] Disinfected by uv-light irradiation.

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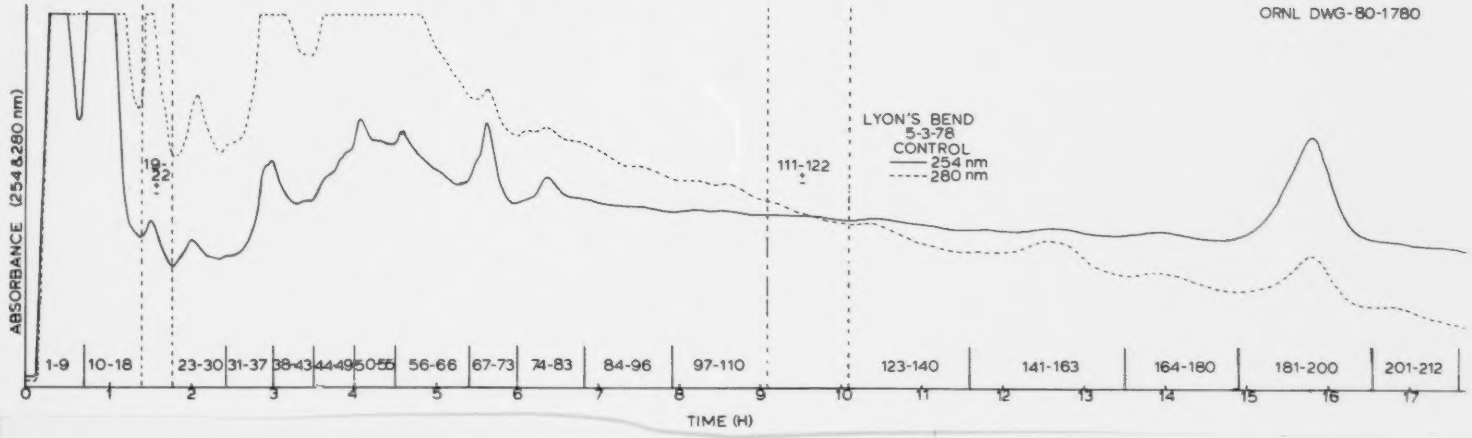

Figure 34. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the unchlorinated LBSE effluent (5/3/78). Mutagenic fractions are shown between dotted lines. The symbol ± means weakly positive mutagenic activity. Fractions 20 and 21 are not shown.

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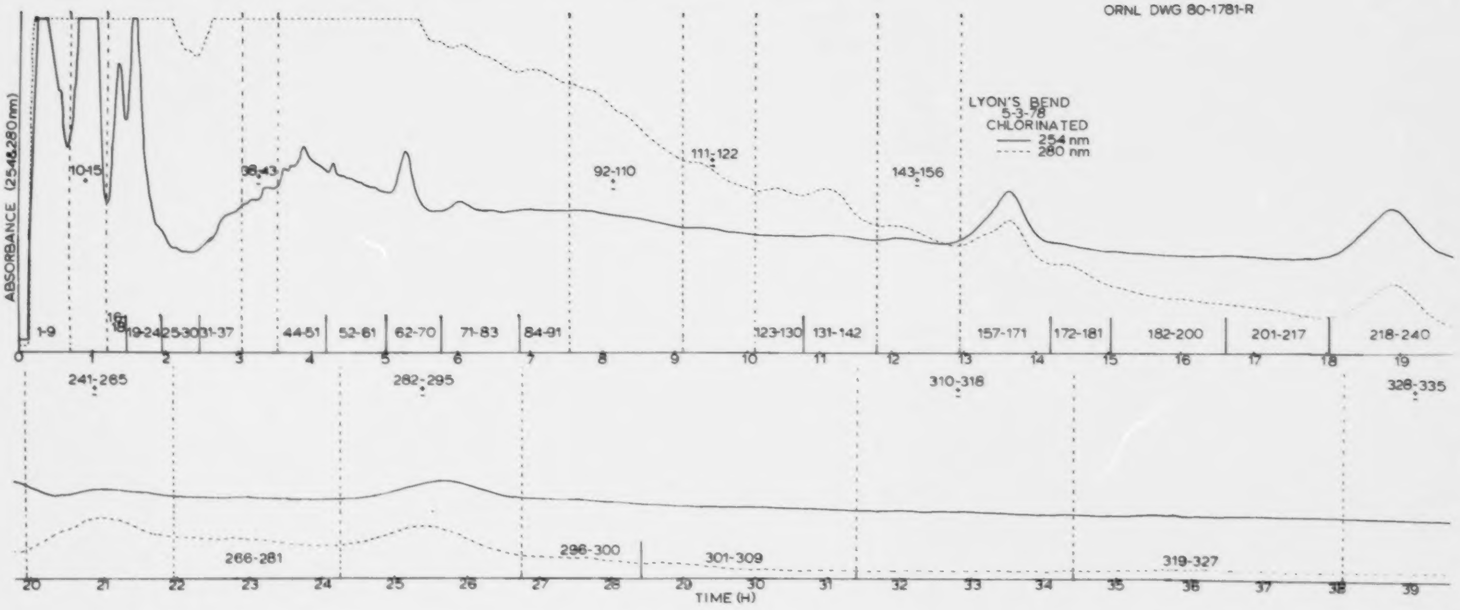

Figure 35. Chromatogram of uv-absorbing constituents in a 1700-fold concentrate of chlorinated LBSE effluent (5/3/78). Mutagenic fractions are shown between dotted lines. The symbol + means positive mutagenic activity and ± means weakly positive mutagenic activity.

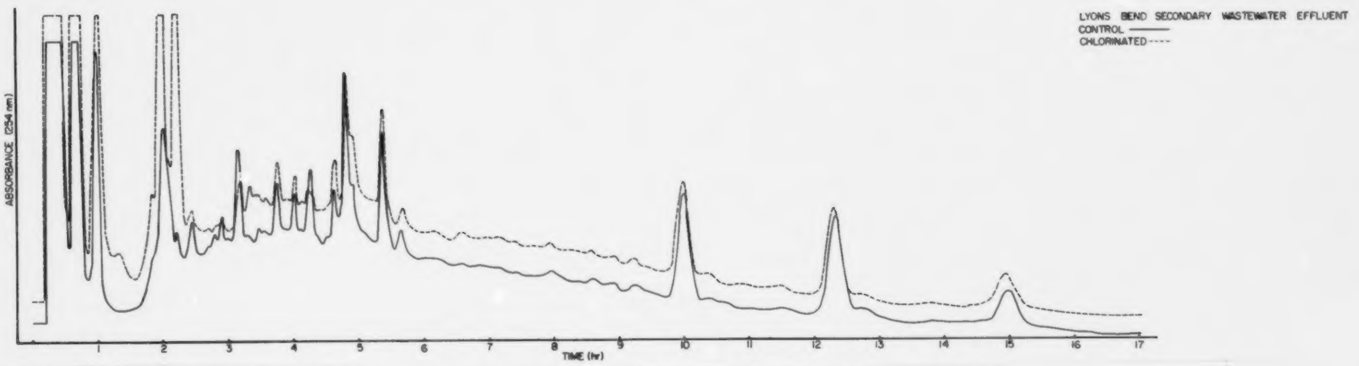

Figure 36. Analytical-scale HPLC chromatograms of 2000-fold concentrates of undisinfected and chlorinated LBSE effluents.

TABLE 17. MUTAGENIC DATA FOR HPLC FRACTIONS, LBSE

Effluent	ID	HPLC fraction no.	Mutagenic activity	TA-1535 Negative control, revertants	TA-1535 Test,[a] revertants	TA-1538 Negative control, revertants	TA-1538 Test,[a] revertants
Set 1							
Control (5/3/78)							
	3	19-22	±	14	26, 37		
	14[b]	111-122	±	25	106, 74, T		
Chlorinated (5/3/78)							
	2	10-15	±	25	40, 136		
	7	38-43	±	25	83, 36, T		
	13	92-100	±	21	82, 26, T		
	14[b]	111-122	±	21	124, 36, T		
	17	143-156	±	21	246, 31, T		
	23	241-265	±	21	41, 39		
	25	282-295	±			11	27, 22
	28	310-318	±			11	25, 28
	30	328-335	±			11	24, 29

[a] First number = revertants/50 µl, second number = revertants/150 µl, T = toxic.
[b] Mutagenic constituents common to both control and disinfected effluents.

the chlorinated Lyons Bend wastewater concentrate noted previously is widely distributed among the organic constituents present. The mutagenicity results are summarized in Table 15.

It was concluded from these results that chlorination of secondary effluents may deactivate some mutagenic constituents (e.g., fraction 19-22, Figure 34), but it may also produce mutagenic constituents (e.g., all fractions except 111-122 in Figure 35). Mutagenic fractions 111-122 were common to both effluents.

Chemical characterization--The mutagenic fractions separated from the unchlorinated and chlorinated effluents were analyzed further in an effort to identify and characterize the mutagenic components in these fractions. Two compounds were identified by GC/MS in the mutagenic fractions separated from the concentrate of the unchlorinated effluent (Table 18 and Figure 34). In addition, four other compounds were tentatively identified by their GC retention position. Twelve compounds were identified by GC/MS, and one compound was tentatively identified by GC retention position in the mutagenic fractions separated from the concentrate of the chlorinated effluent (Table 19 and Figure 35). As typical examples, reconstructed gas chromatograms (RGC) for mutagenic fractions 38-43 and 143-156 with identified compounds labeled are shown in Figures 37 and 38, respectively. Also, the mass spectrum for the unknown constituent identified as 4-chlorobenzoic acid is shown in Figure 39. It should be emphasized that none of the identified constituents are known to be mutagenic.

Set 2--

Chromatograms of 2000-fold concentrates of unchlorinated (control) and chlorinated (residual chlorine, 1.5 mg/L) LBSE effluents (10/25/78) are shown in Figures 40 and 41. Thirty-one pooled HPLC fractions from the control sample and 33 pooled HPLC fractions from the chlorinated effluent were tested for mutagenicity. The majority of the fractions tested positive in both TA-1535 and TA-1538; this clearly indicated problems relative to development of artifacts in the mutagenicity tests. The analysis of the problem will be reported in a later section (p. 150). The conclusion was that the artifacts were caused by reversion of the stock bacterial tester strains to a wild type. Steps were taken to ensure avoidance of the problem thereafter.

Moccasin Bend Wastewater Treatment Plant (MBSE)

Two sets of samples were collected at the Moccasin Bend Wastewater Treatment Plant, Chattanooga, Tennessee, on 9/27/78 and 7/17/79. Each set consisted of unchlorinated (control) and chlorinated effluents. The two sets of samples were concentrated 1000-fold and 2000-fold respectively and examined.

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TABLE 18. IDENTIFIED CONSTITUENTS IN MUTAGENIC HPLC FRACTIONS OF LBSE UNCHLORINATED (CONTROL) SECONDARY EFFLUENT (5/3/78)

Constituent	LC fraction	Identification method[a]
Caffeine	19-22	AC,MS
Phosphate[b]	111-122	GC,MS
Ethylene glycol[c]	111-122	GC
Phenol[c]	111-122	GC
Glycerol[c]	111-122	GC
Adenosine[c]	111-122	GC

[a] AC = anion exchange elution position, GC = gas chromatography retention position, MS = mass spectrum.

[b] Analyzed as the trimethylsilyl (TMS) derivative of phosphate, tris(TMS)PO_4. Phosphate was identified in several chromatographic fractions in most effluents analyzed. It probably represents phosphate hydrolyzed from a phosphorylated organic by the trimethysilylation reagent.

[c] Tentative identifications based only on GC retention positions.

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TABLE 19. IDENTIFIED CONSTITUENTS IN MUTAGENIC HPLC FRACTIONS OF LBSE CHLORINATED SECONDARY EFFLUENT (5/3/78)

Constituent	LC fraction	Identification method[a]
Propylene glycol	10-15	AC,GC,MS
Uracil	10-15	AC,GC,MS
Phenylalanine	10-15	AC,MS
Phosphate[b]	38-43	GC,MS
Diglycolic acid	38-43	MS
α-Glycerophosphate	38-43	MS
3,2-Dimethyl-3-hydroxy butyric acid	38-43	MS
Palmitic acid	38-43	GC,MS
Ethylindan	111-112	MS
Phosphate[b]	111-112	GC,MS
4-Chlorobenzoic acid	143-146	AC,GC,MS
6-Dimethyladenosine[c]	143-146	GC

[a] AC = anion exchange elution position, GC = gsa chromatographic retention position, MS = mass spectrum.

[b] Analyzed as the trimethylsilyl (TMS) derivative of phosphate, tris(TMS)PO_4. Phosphate was identified in several chromatographic fractions in most effluents analyzed. It probably represents phosphate hydrolyzed from a phosphorylated organic by the trimethysilylation reagent.

[c] Tentative identifications based only on GC retention position.

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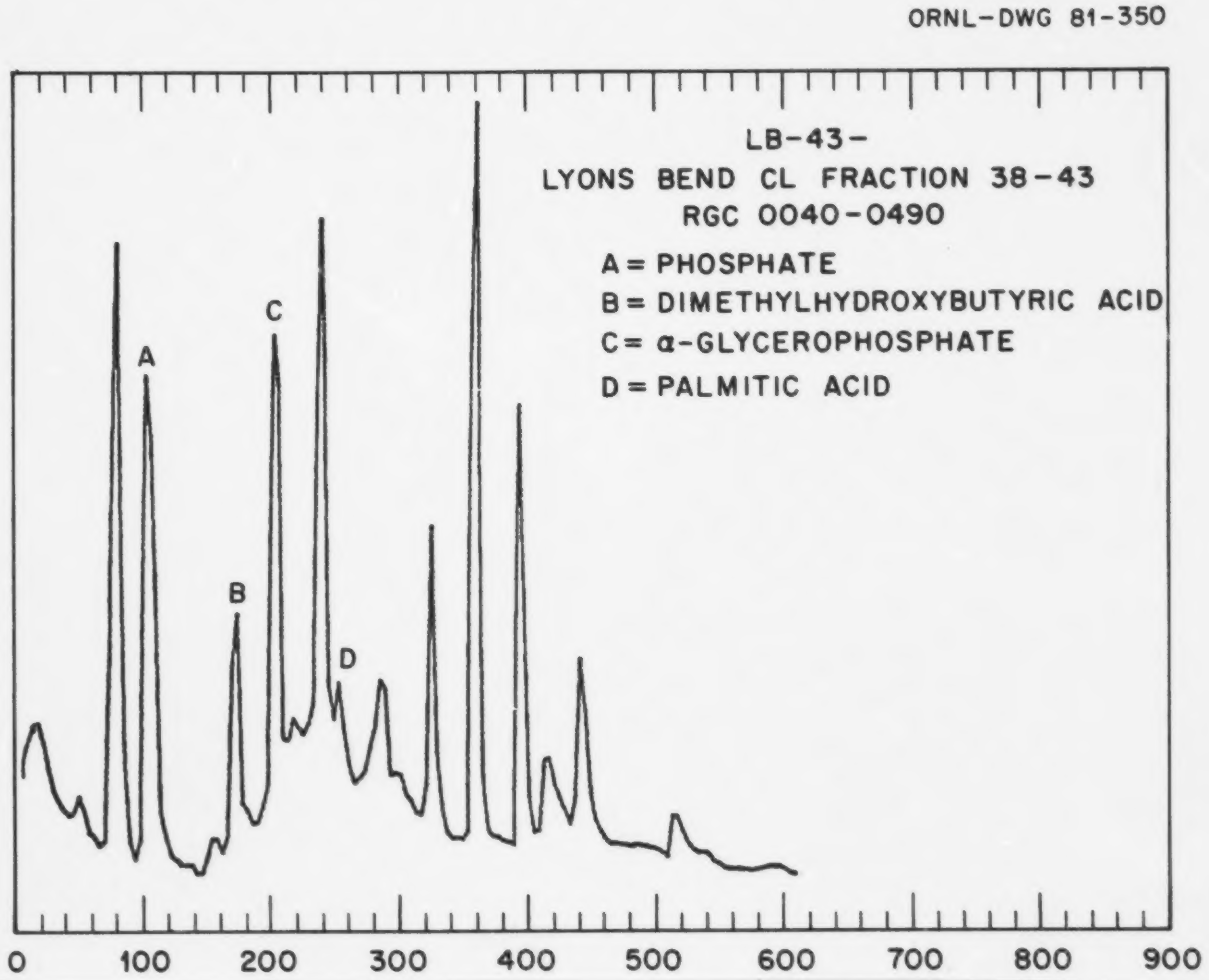

Figure 37. Reconstructed gas chromatogram of HPLC fraction 38-43 with identified constituents labeled.

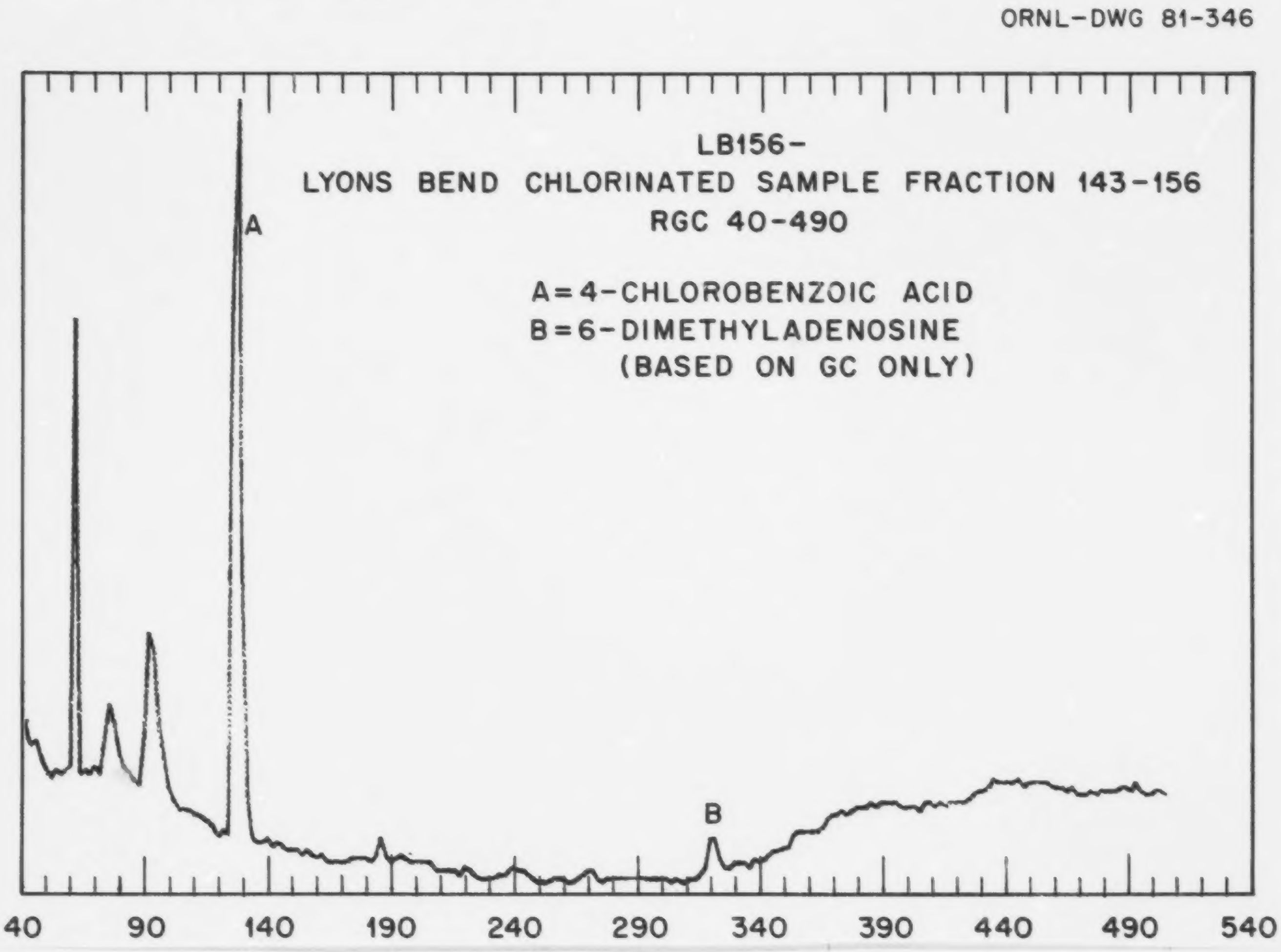

Figure 38. Reconstructed gas chromatogram of HPLC fraction 143-156 with identified constituents labeled.

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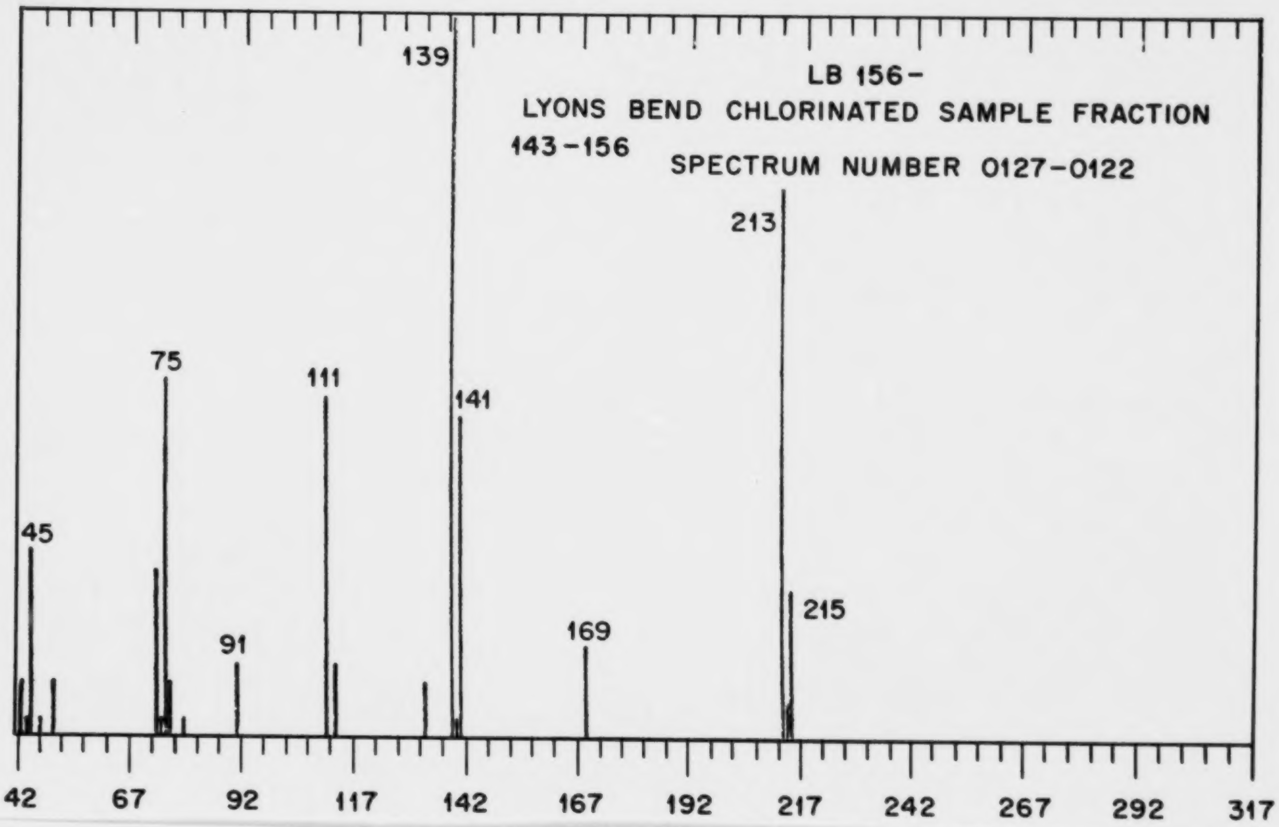

Figure 39. GC/MS scan 127 constituent identified as 4-chlorobenzoic acid.

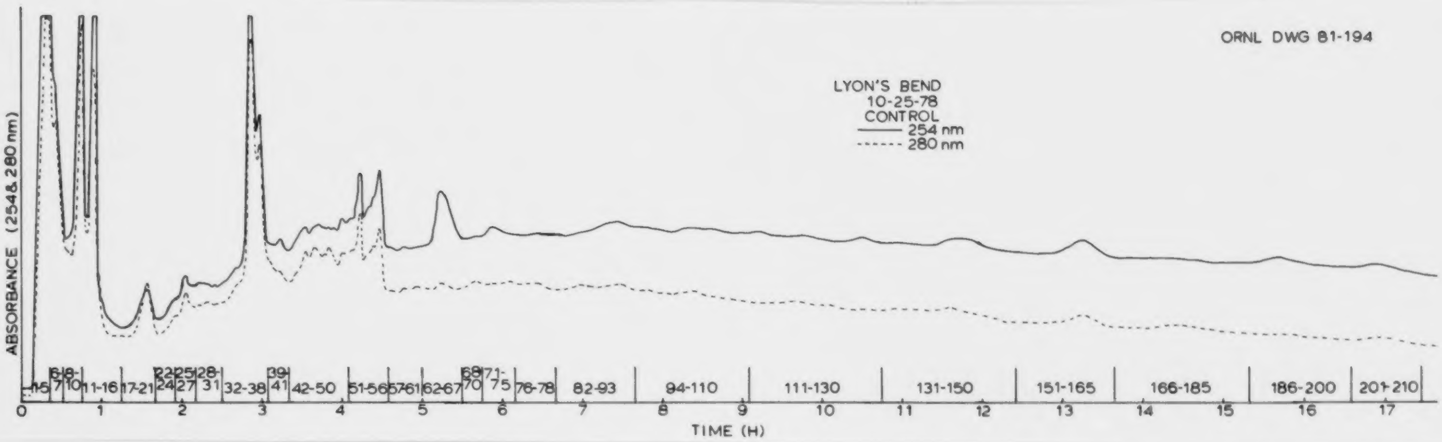

Figure 40. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the unchlorinated LBSE effluent (10/25/78). Fractions 26 to 31 are not shown.

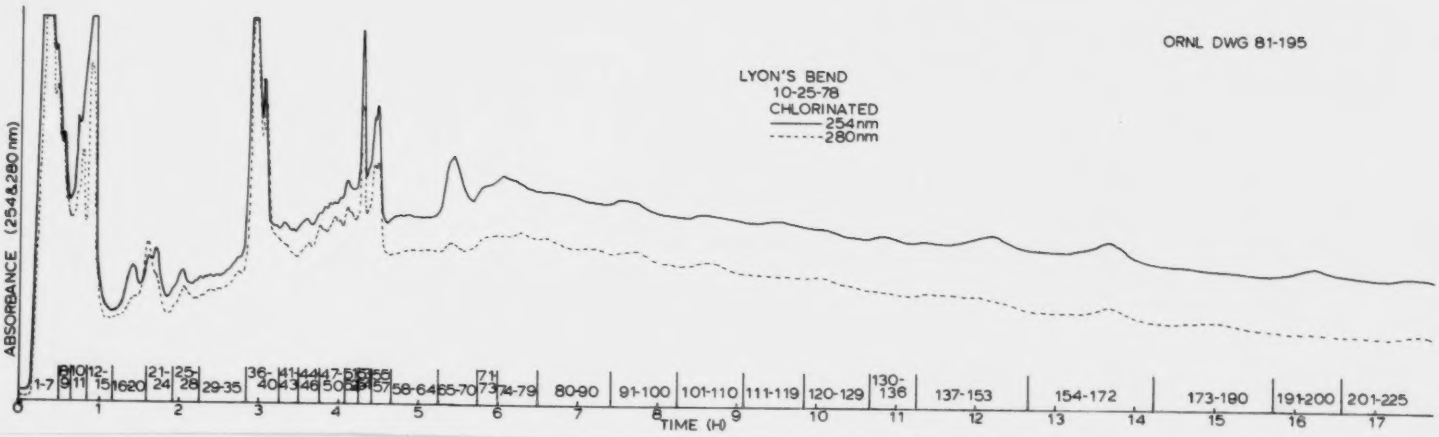

Figure 41. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the chlorinated LBSE effluent (10/25/78). Fractions 31 to 33 are not shown.

Set 1--

Mutagenicity--The concentrate of undisinfected (control) effluent (9/27/78) was weakly mutagenic; however, the concentrate of the chlorinated effluent was not mutagenic (Table 11). Because the lack of mutagenicity in the concentrate of the chlorinated effluent appeared to be due to toxicity, the sample was retested at a 500-fold concentration. In this retest the weakly mutagenic unchlorinated sample was found to be strongly positive. The chlorinated sample still showed little mutagenic activity but under conditions which supported the interpretation of toxicity to the tester strains. Figures 42 and 43 show the chromatograms of the uv-absorbing constituents in these samples and the eluate fractions found to possess mutagenic activity.

Twenty-five samples representing 282 HPLC fractions from the unchlorinated sample were tested with TA-1535 and TA-1538. Of the 25 samples, one, representing fractions 101-115, was positive in TA-1535. Six other samples showed slight but clearly positive results in the same strain, and two additional samples were slightly positive in TA-1538. The positive results from several fractions of the unchlorinated concentrate were consistent with our previous finding that the unchlorinated Moccasin Bend concentrate was more mutagenic than that of the same wastewater following chlorination. In this case, the separated fractions had more total mutagenic activity than the original concentrate. The mutagenic eluate fractions are listed in Table 20 and are shown in Figure 42.

Thirty-two pooled fractions derived from 296 HPLC eluate fractions from the concentrate of the chlorinated wastewater were tested with TA-1535 and TA-1538. Of these, two were slightly positive in TA-1535 and seven others were slightly positive in TA-1538. The original concentrate from which these fractions were derived was negative in both strains when tested at 1000-fold concentration. Consequently, this represents another case where mutagenicity increases with fractionation--probably due to separation of toxic from mutagenic components. The total mutagenicity in the fractions derived from the chlorinated concentrate is less than that of the fractions derived from the concentrate of the unchlorinated Moccasin Bend wastewater. This is in agreement with the greater mutagenicity of the unchlorinated concentrate. The mutagenic eluate fractions are listed in Table 20 and shown in Figure 43. The HPLC mutagenicity test results are summarized in Table 15.

Chemical characterization--Efforts were made to identify the mutagenic constituents in the HPLC fractions from these samples. Although characterization of constituents in the fractions found to have mutagenic activity was emphasized, several other nonmutagenic fractions were examined also. Twelve constituents were identified in the unchlorinated and 15 identified in chlorinated effluent

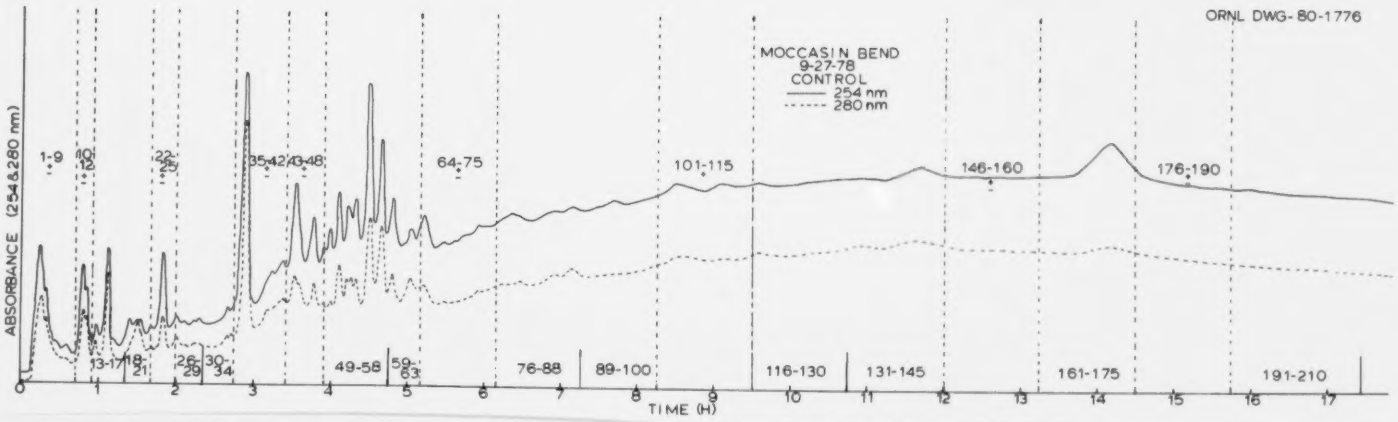

Figure 42. Chromatogram of uv-absorbing constituents in a 1000-fold concentrate of the unchlorinated MBSE effluent (9/27/78). Mutagenic fractions are shown between dotted lines. The symbol + means positive mutagenic activity and ± means weakly positive mutagenic activity. Fractions 22 to 25 are not shown.

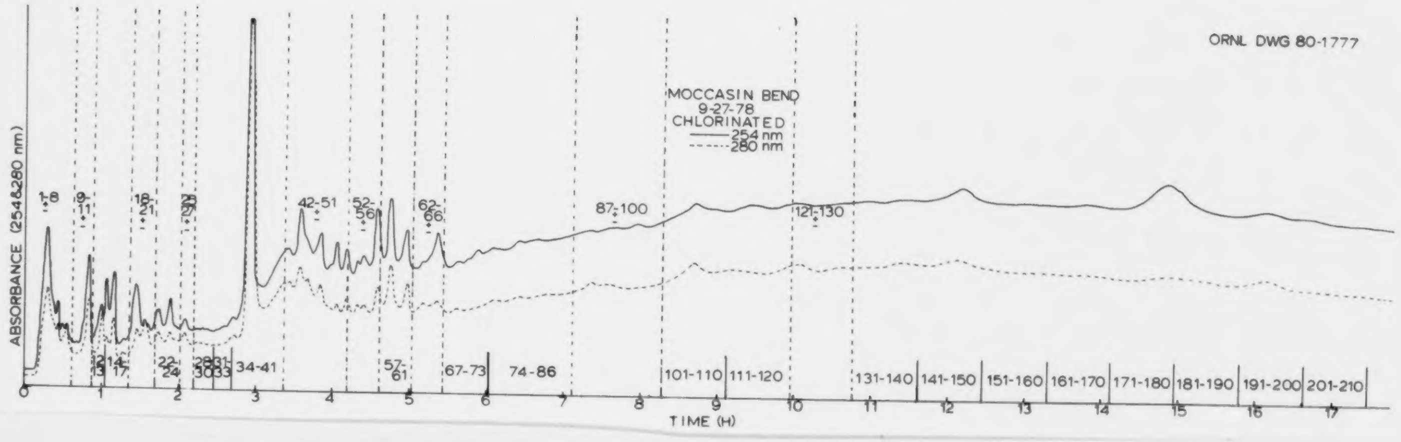

Figure 43. Chromatogram of uv-absorbing constituents in a 1000-fold concentrate of the chlorinated MBSE effluent (9/27/78). Mutagenic fractions are shown between dotted lines. The symbol ± means weakly positive mutagenic activity. Fractions 29 to 32 are not shown.

TABLE 20. MUTAGENIC DATA FOR HPLC FRACTIONS, MBSE

				TA-1535		TA-1538	
Effluent	ID	HPLC fraction no.	Mutagenic activity	Negative control, revertants	Test,[a] revertants	Negative control, revertants	Test,[a] revertants
Set 1							
Control (9/27/78)	1^b	1-9	±	33	110, 51, T		
	2^b	10-12	±	33	77, 68		
	5^b	22-25	±			33	57, 70
	8	35-42	±			33	49, 63
	9^b	43-48	±	33	37, 72		
	12	64-75	±	26	37, 54		
	15	101-115	+	26	69, 269		
	18	146-160	±	26	33, 55		
	20	170-190	±	26	66, 55		
Chlorinated (9/27/78)	1^b	1-8	±			42	61, 81
	2^b	9-11	±			42	59, 87
	5^b	18-21	±	20	20, 51		
	7	25-27	±			42	52, 79
	11^b	42-51	±			22	23, 43
	12	52-56	±	20	47, 50		
	14	62-66	±			22	44, 41
	17	87-100	±			22	41, 57
	20	121-130	±			22	29, 41

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TABLE 20. (continued)

Effluent	ID	HPLC fraction no.	Mutagenic activity	TA-1535 Negative control, revertants	TA-1535 Test,[a] revertants	TA-1538 Negative control, revertants	TA-1538 Test,[a] revertants
Set 2							
Control (7/17/79)	2	7-10	+			7	36, 8, T
	4	15-16	±			7	16, 10
	5	17-19	±			7	9, 18
	6	20-22	±			7	19, 12
	11[b]	35-47	±			7	18, 5
	12	48-57	±	8	12, 14		
	13[b]	58-67	±	8	14, 4, T	7	14, 13
	14	68-73	±			7	4, 24
	15	74-78	±			7	14, 5, T
Chlorinated (7/17/79)	8[b]	32-47	±			18	30, 37
	10[b]	60-62	±	29	42, 80		
	21	201-225	+	12	55, 90	15	24, 84

[a] First number = revertants/50 μL, second number = revertants/150 μL, T = toxic.

[b] Common to both effluents.

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concentrates (Tables 21 and 22). The constituents appear to be metabolic products and other compounds anticipated in wastewater effluents.[6] The appearance of benzoic acid, lactic acid, and several fatty acids in several HPLC fractions cannot be explained. They are perhaps fragments of larger molecules that are hydrolyzed during the derivatization process.

Set 2--

Mutagenicity--The concentrates of the unchlorinated (control) and chlorinated effluents (7/17/79) were weakly and strongly mutagenic to TA-1535, respectively (Table 11). Figures 44 and 45 show the chromatograms of the uv-absorbing constituents separated from the effluent concentrates and the fractions found to have mutagenic activity.

Initial bacterial mutagenicity testing of the control and chlorinated effluent fractions yielded a large number of positives indicating possible sample contamination. The testing for the MBSE control and chlorinated effluent fractions were repeated using a modified bacterial testing procedure requiring filtration of all samples and strict quality assurance controls. The fractions of the undisinfected sample were one out of 31 (1/31) positive in TA-1538, and 2/31 and 7/31 slightly positive in TA-1535 and TA-1538, respectively (Table 20 and Figure 44). These results show the new mutagenic test procedure to be satisfactory, with a high probability that the test results are accurate.

Twenty-two fractions of the chlorinated effluent concentrate were retested using the modified bacterial test procedure. One of the 22 fractions was positive in both TA-1535 and TA-1538 strains, one was slightly positive in TA-1535, and another showed a slightly positive effect in TA-1538 (Table 20 and Figure 45).

Mutagenic data for the effluent samples is summarized in Table 15.

Chemical characterization--Nine constituents were identified in the unchlorinated and seven identified in the chlorinated effluent concentrates (Tables 23 and 24).

Again the same pattern for distribution of fatty acids (e.g., palmitic acid) was observed. Note that phosphate was commonly found in these effluents as compared with the absence of phosphate in the 9/27/78 effluents. An interesting compound, 2-methyl-3-propyl oxirane, belonging to the commonly mutagenic epoxide family was tentatively identified by mass spectral comparison with the computer library. Efforts were made to obtain the compound to confirm the identification and to test for mutagenicity. It is not commercially available but may be custom synthesized.

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TABLE 21. IDENTIFIED CONSTITUENTS IN HPLC FRACTIONS OF MBSE UNCHLORINATED (CONTROL) SECONDARY EFFLUENT (9/27/78)

Constituent	HPLC fraction	Identification method[b]
Serine	10-12[a]	GC
Palmitic acid	10-12[a]	GC, MS
Benzoic acid	18-21	GC, MS
Palmitic acid	18-21	GC, MS
Myristic acid	18-21	GC
Lactic acid	22-25[a]	GC, MS
Oxalic acid	22-25[a]	GC, MS
Erythritol	22-25[a]	GC
Glutamic acid	22-25[a]	GC
Caffeine	22-25[a]	LC, MS
Palmitic acid	22-25[a]	MS
Alanine	26-29	GC, MS
Stearic acid	26-29	GC, MS
Benzoic acid	35-42[a]	GC, MS
Lactic acid	35-42[a]	GC, MS
Cyclohexane-1,4-diol	35-42[a]	GC

[a] Mutagenic fraction.

[b] LC = HPLC chromatographic retention time, GC = gas chromatographic retention time, MS = mass spectra.

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TABLE 22. IDENTIFIED CONSTITUENTS IN HPLC FRACTIONS OF MBSE CHLORINATED (CHLORINE RESIDUAL <0.2 mg/L) SECONDARY EFFLUENT (9/27/78)

Constituent	LC fraction	Identification method[a]
Alanine	9-11[b]	GC
3-Hydroxyisovaleric acid	9-11[b]	GC
Aminoisobutyric acid	12-13	GC
Benzoic acid	12-13	GC, MS
Alanine	12-13	GC
Canavanine	12-13	GC
Palmitic acid	12-13	GC, MS
Stearic acid	12-13	GC, MS
Benzoic acid	18-21[b]	GC, MS
Inositol	18-21[b]	GC, MS
Palmitic acid	18-21[b]	MS
Diglycolic acid	25-27[b]	GC, MS
Lactic acid	25-27[b]	GC, MS
Muconic acid	25-27[b]	GC
3-Hydroxyisovaleric acid	25-27[b]	GC, MS
Glycerine	25-27[b]	GC, MS
Xylose	25-27[b]	GC
2-Methylxylopyranoside	25-27[b]	GC, MS
Palmitic acid	25-27[b]	GC, MS
Linoleic acid	25-27[b]	GC
Stearic acid	25-27[b]	GC, MS
Lactic acid	34-41	MS

[a] GC = gas chromatographic retention position, MS = mass spectra.
[b] Mutagenic fraction.

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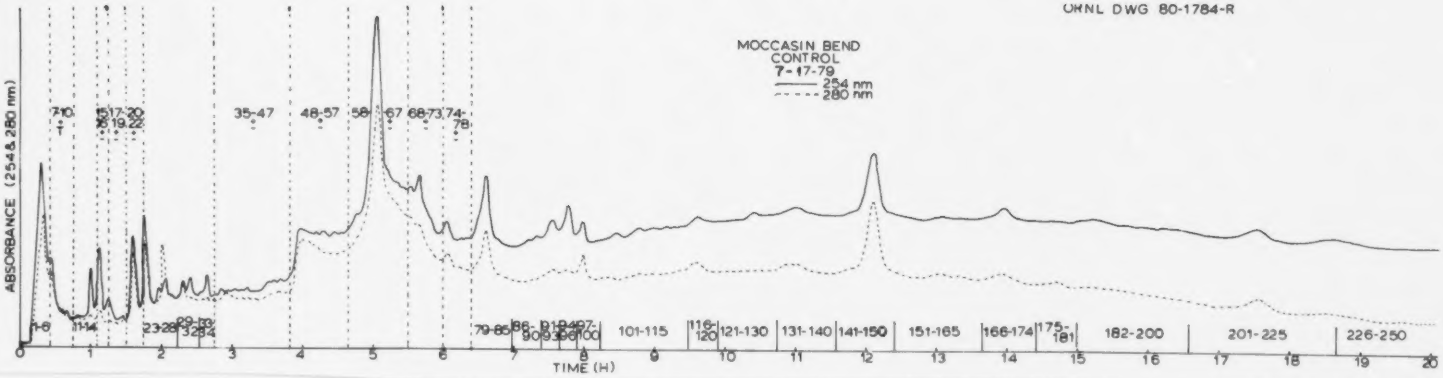

Figure 44. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the unchlorinated MBSE effluent (7/17/79). Mutagenic fractions are shown between dotted lines. The symbol + means positive mutagenic activity, ± means weakly positive, and T means toxic. Fraction 31 is not shown.

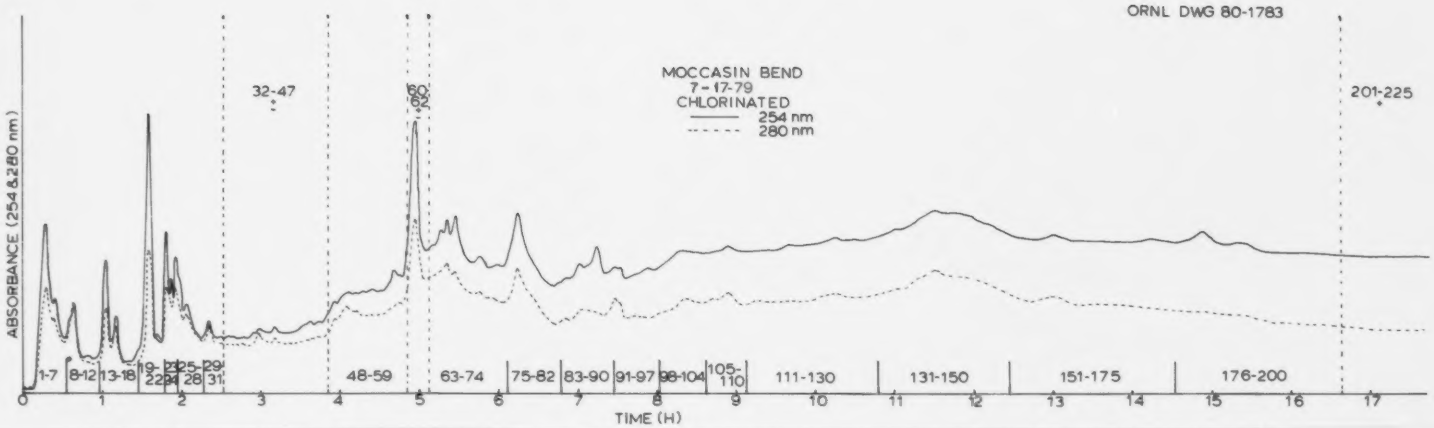

Figure 45. Chromatogram of the uv-absorbing constituents in a 2000-fold concentrate of the chlorinated MBSE effluent (7/17/79). Mutagenic fractions are shown between dotted lines. The symbol + means positive mutagenic activity and ± means weakly positive. Fraction 22 is not shown.

TABLE 23. IDENTIFIED CONSTITUENTS IN MUTAGENIC HPLC FRACTIONS OF MBSE UNCHLORINATED (CONTROL) SECONDARY EFFLUENT (7/17/79)

Constituent	LC fraction	Identification method[a]
Palmitic acid	17-19	GC, MS
Dodecane	17-19	MS
2-Methyloctane	17-19	MS
2,5-Dimethyl-3,4-hexanediol	20-21	MS
2,2,3,3-Tetramethylhexane	20-21	MS
Phosphate[b]	35-47	GC, MS
Diisobutylphthalate	35-47	MS
Lactic acid	48-57	GC, MS
Phosphate[b]	48-57	GC, MS
Diisobutylphthalate	48-57	GC
Phosphate[b]	58-67	GC, MS
2,4-Dimethyl-3-pentanol	68-73	MS
Phosphate[b]	68-73	GC, MS
Phosphate[b]	74-78	GC, MS
Palmitic acid	74-78	GC, MS

[a] GC = gas chromatographic retention time, MS = mass spectral identification.

[b] Analyzed as the trimethylsilyl (TMS) derivative of phosphate, tris (TMS) PO_4. Phosphate was identified in several chromatographic fractions in most effluents analyzed. It probably represents phosphate hydrolyzed from a phosphorylated organic by the trimethysilylation reagent.

TABLE 24. IDENTIFIED CONSTITUENTS IN MUTAGENIC FRACTIONS OF MBSE CHLORINATED (CHLORINE RESIDUAL, <0.2 MG/L) SECONDARY EFFLUENT (7/17/79)

Constituent	LC fraction	Identification method[a]
Alanine	32-47	MS
Benzoic acid	32-47	MS
Phosphate[b]	32-47	MS
Phosphate[b]	60-62	MS
Palmitic acid	60-62	MS
Phosphate[b]	201-205	MS
Palmitic acid	201-205	MS
Stearic acid	201-205	MS
2-Methyl-3-propyl oxirane	201-205	MS
4-Butoxy-1-butanol	201-205	MS

[a] MS = mass spectral identification.

[b] Analyzed as the trimethylsilyl (TMS) derivative of phosphate, tris(TMS)PO_4. Phosphate was identified in several chromatographic fractions in most effluents analyzed. It probably represents phosphate hydrolyzed from a phosphorylated organic by the trimethysilylation reagent.

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Marlborough Easterly Advanced Waste Treatment Plant (MMSE)

Two sets of samples were collected at the Marlborough Easterly Advanced Waste Treatment Plant, Marlborough, Massachusetts on 3/12/79 and 9/19/79. Each set included undisinfected (control), ozonated, and chlorinated effluents. The effluent samples were concentrated 2000-fold and examined.

Set 1--

Mutagenicity--No mutagenic activity was detected in the concentrates of both the ozonated and chlorinated effluents (Table 11). These results suggest that the disinfection processes destroys some mutagenic substances present in the undisinfected effluent because the concentrate of the undisinfected effluent (3/12/79) was mutagenic in both TA-1535 and TA-1538. Figures 46, 47, and 48 show the chromatograms of uv-absorbing constituents in the concentrates of the control, ozonated, and chlorinated effluents, respectively. The mutagenic fractions are also shown in the figures.

Thirty-one pooled fractions representing 270 HPLC fractions from the undisinfected (control) effluent were tested for mutagenicity. Only one fraction was slightly positive in TA-1538 (Figure 46 and Table 25) although the concentrate before chromatography was mutagenic with both TA-1535 and TA-1538.

Sixteen fractions collected from the ozonated effluent concentrate were tested. Four were positive and one was slightly positive in both strains (Figure 47 and Table 25). This pattern indicated possible development of artifacts in bacterial mutagenicity. No mutagenic activity was detected in the effluent concentrate before chromatography.

Of the 24 pooled fractions collected from the chlorinated effluent concentrate, two were positive (Figure 48 and Table 25). No mutagenic activity was detected in the effluent concentrate before chromatography.

The HPLC mutagenicity test results are summarized in Table 15.

The relative lack of mutagenic activity in the chromatographic fractions separated from the mutagenic concentrates of the undisinfected effluent supports the conclusion that some mutagenic components may be deactivated by HPLC. The presence of mutagenic activity in the chromatographic fractions separated from the apparently nonmutagenic concentrate of the chlorinated effluent tends to support the conclusion that the mutagenicity of some components may be masked or hidden when the compound is in complex mixtures.

Chemical characterization--The constituents after ozonation (Figure 47) and after chlorination (Figure 48) for the MMSE nitrified

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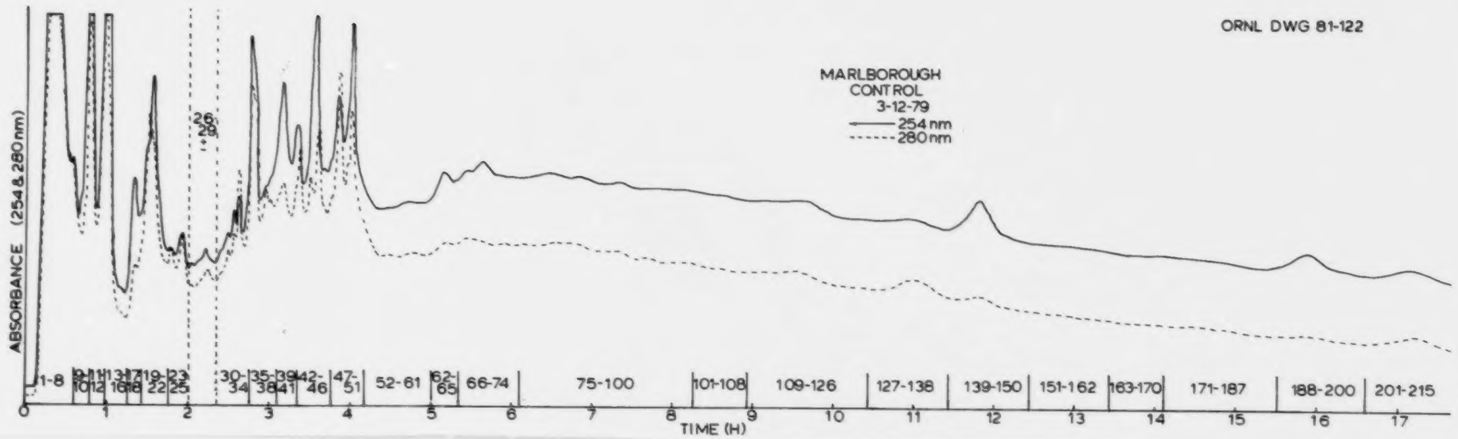

Figure 46. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the undisinfected (control) MMSE effluent (3/12/79). The mutagenic fraction is shown between dotted lines. The symbol ± means weakly positive mutagenic activity. Fractions 27 to 31 are not shown.

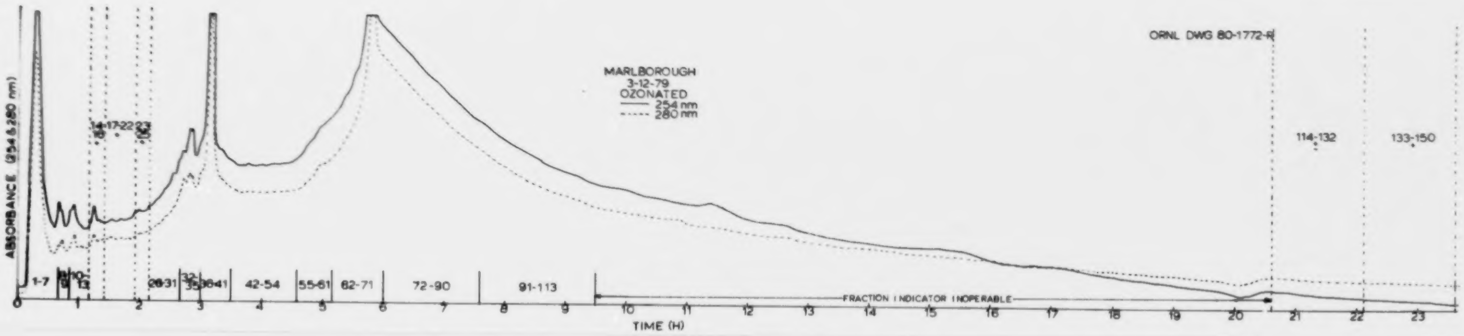

Figure 47. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the ozonated MMSE effluent (3/12/79). The mutagenic fractions are shown between dotted lines. The symbol + means positive mutagenic activity and ± means weakly positive.

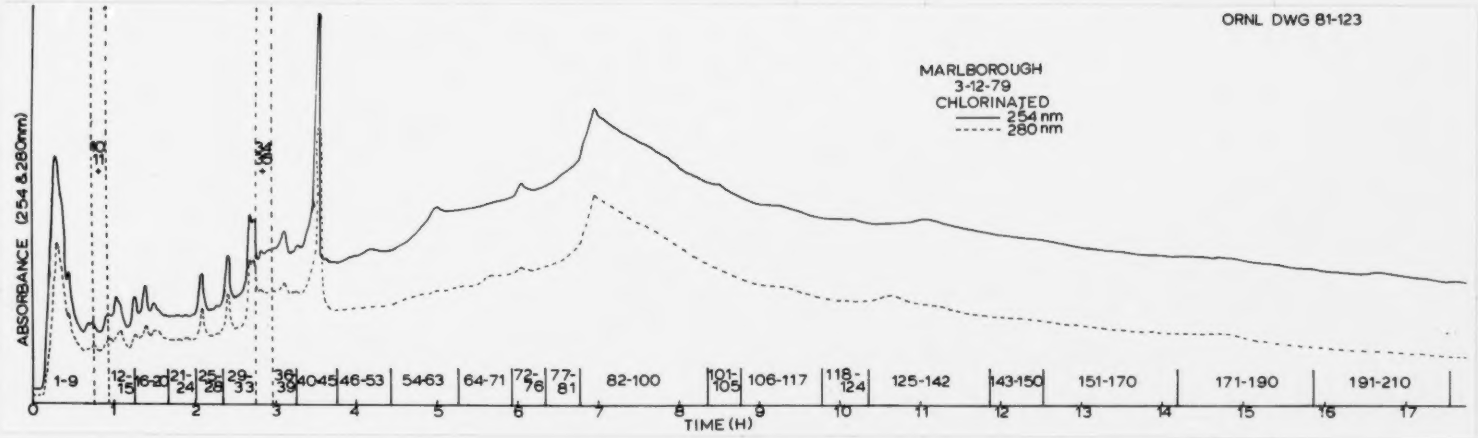

Figure 48. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the chlorinated MMSE effluent (3/12/79). The mutagenic fractions are shown between dotted lines. The symbol + means positive mutagenic activity.

TABLE 25. MUTAGENIC DATA FOR HPLC FRACTIONS, MMSE

Effluent	ID	HPLC fraction no.	Mutagenic activity	TA-1535 Negative control, revertants	TA-1535 Test,[a] revertants	TA-1538 Negative control, revertants	TA-1538 Test,[a] revertants
Set 1							
Control (3/12/79)	8	26-29	±			11	16, 22
Ozonated (3/12/79)	4	14-16	+	13	128, 152	15	90, 422
	5	17-22	+	13	136, 165	15	272, 400
	6	23-25	+	13	122, 500	15	500, 760
	15	114-132	+	13	27, 79	15	27, 128
	16	133-150	±	13	15, 36	15	29, 71
Chlorinated (3/12/79)	3	10-11	+	24	200, 250	52	150, 200
	10	34-35	+			52	400, 83, T
Set 2							
Control (9/19/79)	13	82-100	±			15	27, 13, T
	14	101-113	±			15	44, 35, T
	15	114,120	±	12	24, 14, T		
	16	121-127	±	12	20, 25		
	18	134-140	±	12	12, 27		
	20	151-175	±	12	21, 26		
	21	176-200	±	12	42, 50	15	18, 30
	22	201-225	±	12	22, 35	15	20, 32
	23	226-250	±			15	34, 29, T
Ozonated (9/19/79)	18	126-150	±	20	30, 40		
	20	176-200	±			18	36, 34

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TABLE 25. (continued)

Effluent	ID	HPLC fraction no.	Mutagenic activity	TA-1535 Negative control, revertants	TA-1535 Test,[a] revertants	TA-1538 Negative control, revertants	TA-1538 Test,[a] revertants
Chlorinated (9/19/79)	2	12-14	±	25	22, 52		
	5	22-24	±			16	34, 22, T
	9	40-47	±			16	27, 46
	11	52-56	+	25	110, 250	16	163, 300
	13	66-70	+	25	57, 97	16	53, 119
	18	151-175	+			16	48, 270
	20	201-225	±			16	31, 35
	21	226-250	±			16	32, 25, T
	22	251-275	+	25	283, 400	16	241, 350

[a] First number = revertants/50 µL, second number = revertants/150 µL, T = toxic.

effluents were compared with the constituents in the MMSE nitrified effluent prior to disinfection (Figure 46). Many chromatographic peaks in the control sample are missing in both the ozonated and chlorinated effluents, which are quite similar in appearance. This reduction of chromatographic peaks parallels the reduction of mutagenicity during disinfection by ozonation and chlorination.

Efforts were made with limited success to characterize constituents in the mutagenic fractions of the ozonated effluents (Table 26).

Set 2--

Mutagenicity--Series of 23 and 24 pooled fractions from the HPLC chromatography of the undisinfected and ozonated MMSE effluents, respectively, were tested for bacterial mutagenicity in TA-1535 and TA-1538 (Figures 49 and 50). The MMSE control had six slightly positive fractions in the base pair substitution detector *Salmonella* strain TA-1535 and five slightly positive fractions in the frame-shift detector strain TA-1538 (Table 25). In contrast, only one slightly positive fraction was found for both TA-1535 and TA-1538 in the testing of the MMSE ozonated effluent (Table 25). This data corroborates the observation that ozonation destroys some mutagenic substances present in the undisinfected MMSE effluent.

Of the 23 pooled fractions resulting from chromatography of the chlorinated effluent concentrate, two were strongly positive and two slightly positive in TA-1535, whereas four were positive and four slightly positive in TA-1538 (Table 25 and Figure 51). Chlorination of the effluent appears to have destroyed some of the mutagenic components found in the control concentrate. On the other hand chlorination apparently produced several mutagenic constituents.

All bacterial mutagenicity tests for Set 2 were made with the modified bacterial testing procedure and, thus, should be free of false positive artifacts.

The mutagenic data for HPLC fractions for Set 2 are summarized in Table 15.

Chemical characterization--Twenty-two constituents were identified in the concentrate of the control MMSE nitrified effluent prior to disinfection (Table 27). Phosphate is the only component that was identified in every mutagenic HPLC fraction. As in several other HPLC separations, a multiplicity of such constituents as fatty acids, lactic acid, etc. was observed.

Thirty-one constituents were identified in the concentrate of the nitrified, ozonated MMSE effluent (Table 28). None of the compounds identified in the mutagenic HPLC fractions are believed to be mutagenic.

TABLE 26. IDENTIFIED CONSTITUENTS IN MUTAGENIC HPLC FRACTIONS OF MMSE OZONATED EFFLUENT (3/12/79)

Constituent	LC fraction	Identification method[a]
Phosphate[b]	14-16	GC, MS
1,3-Propanediol	17-22	MS
Lactic acid	23-25	MS
2-Methyl-2-hydroxybutyric acid	23-25	MS
Phosphate[b]	114-132	GC, MS
Phosphate[b]	133-150	GC, MS

[a] GC = gas chromatographic retention times, MS = mass spectra comparison.

[b] Analyzed as the trimethylsilyl (TMS) derivative of phosphate, tris(TMS)PO_4. Phosphate was identified in several chromatographic fractions in most effluents analyzed. It probably represents phosphate hydrolyzed from a phosphorylated organic by the trimethysilylation reagent.

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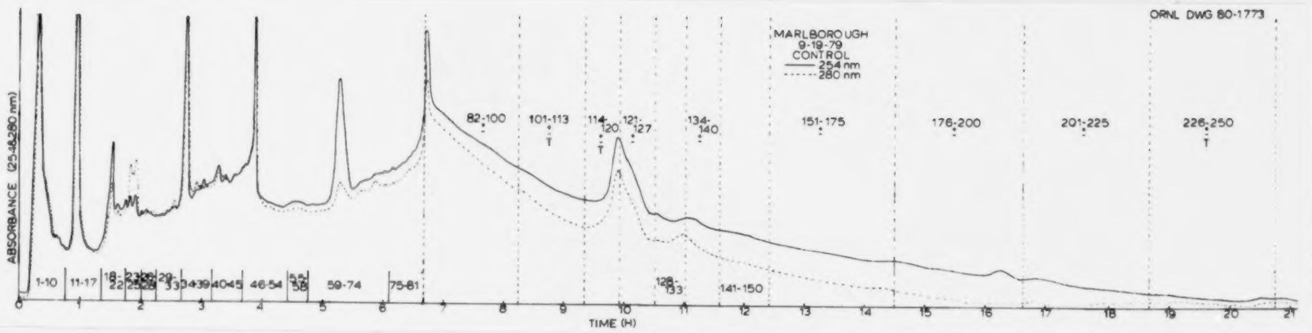

Figure 49. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the undisinfected MMSE effluent (9/19/79). The mutagenic fractions are shown between dotted lines. The symbol ± means slightly positive mutagenic activity and T means toxic.

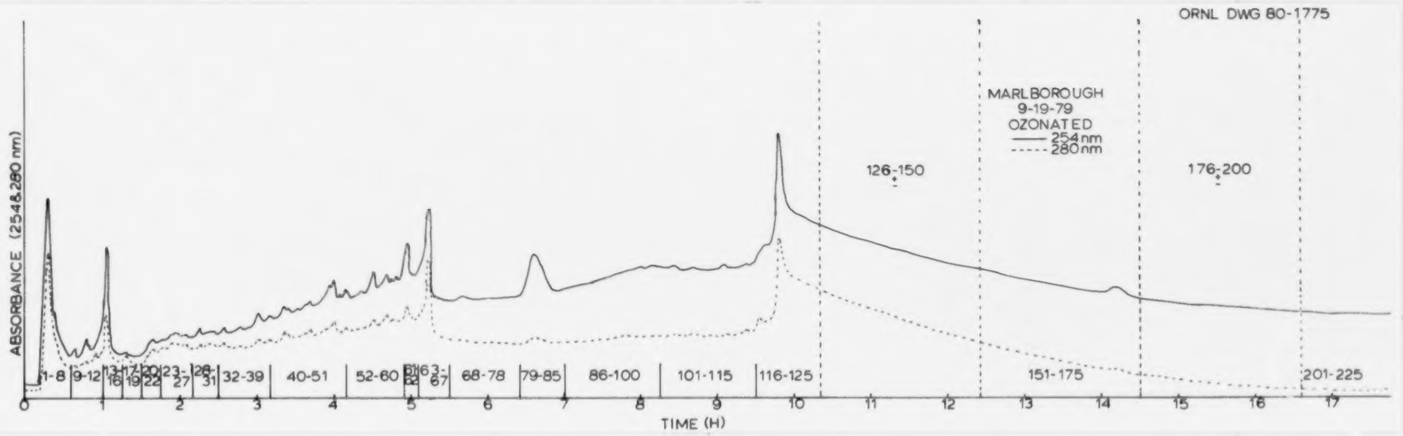

Figure 50. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the ozonated MMSE effluent (9/19/79). The mutagenic fractions are shown between dotted lines. The symbol ± means weakly positive mutagenic activity. Fractions 22 to 24 are not shown.

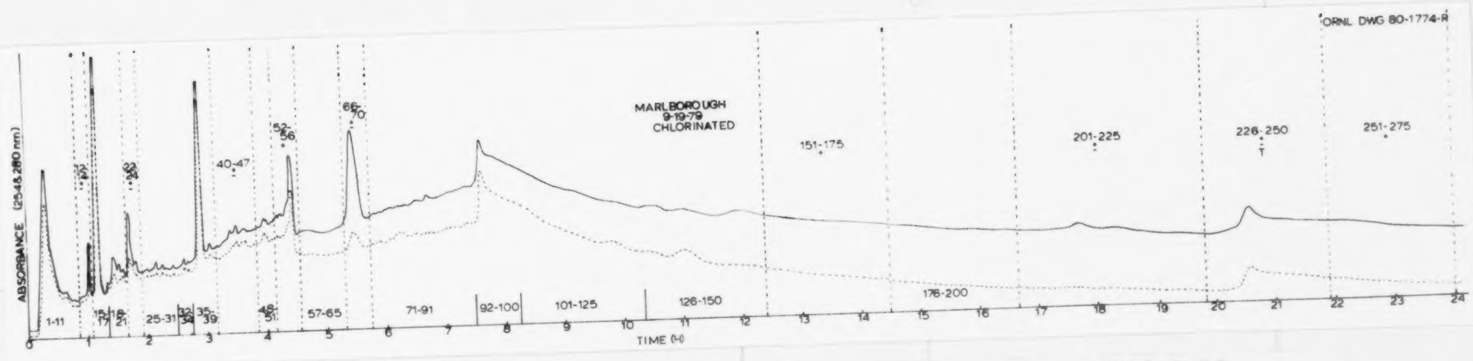

Figure 51. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the chlorinated MMSE effluent (9/19/79). The mutagenic fractions are shown between dotted lines. The symbol + means positive mutagenic activity and ± means weakly positive. Fraction 23 is not shown.

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TABLE 27. IDENTIFIED CONSTITUENTS IN HPLC FRACTIONS OF MMSE UNDISINFECTED (CONTROL) EFFLUENT (9/19/79)

HPLC fraction/constituent	Identification method[a]
12-17	
Benzoic acid	MS
Dihydroxy acetone	GC, MS
4-Chlorobenzoic acid	MS
Threitol	MS
Glycerol	MS
Di-n-Butylphthalate	MS
18-22	
Threitol	MS
Benzoic acid	MS
Phosphate[b]	GC, MS
4-Chlorobenzoic acid	MS
Glucitol	MS
Palmitic acid	MS
Oleic acid	MS
Stearic acid	MS
23-25	
Erythrose	MS
Phosphate[b]	GC, MS
Threono-1,4-lactone	MS
Glucitol	MS
Palmitic acid	MS
26-33	
2-Hydroxybutyric acid	MS
Phosphate[b]	GC, MS
34-36	
Lactic acid	MS
1,3-Propanediol	MS
Phosphate[b]	GC, MS
Threitol	MS
Palmitic acid	MS
Stearic acid	MS
Tetradecanoic acid	MS
5-Methyl-5-ethyl decane	MS
37-43	
Phosphate[b]	GC, MS
Xylose	MS
44-50	
Malonic acid	MS
Benzoic acid	MS
Phosphate[b]	GC, MS
4-Chlorobenzoic acid	MS
Glucitol	MS
Diisobutylphthalate	MS
63-68	
Urea	MS
Phosphate[b]	GC, MS
Glucitol	MS
81-85	
4-Butoxy-1-butanol	MS
82-100[c]	
Phosphate[b]	GC, MS
101-113[c]	
Phosphate[b]	GC, MS
114-120[c]	
Phosphate[b]	GC, MS
121-127[c]	
Phosphate[b]	GC, MS
131-140[c]	
Phosphate[b]	GC, MS
151-175[c]	
Phosphate[b]	GC, MS
176-200[c]	
Phosphate[b]	GC, MS
201-225[c]	
Phosphate[b]	GC, MS

[a] GC = gas chromatographic retention time, MS = mass spectra comparison.

[b] Analyzed as the trimethylsilyl (TMS) derivative of phosphate, tris(TMS)PO_4. Phosphate was identified in several chromatographic fractions in most effluents analyzed. It probably represents phosphate hydrolyzed from a phosphorylated organic by the trimethysilylation reagent.

[c] Mutagenic fraction.

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TABLE 28. IDENTIFIED CONSTITUENTS IN HPLC FRACTIONS OF MMSE OZONATED, NITRIFIED EFFLUENTS (9/19/79)

HPLC fraction/constituent	Identification method[a]
9-12	
Uracil	MS
2,2-Dimethyl-3-hydroxybutyric acid	MS
Threitol	MS
13-16	
Lactic acid	MS
Phosphate[b]	GC, MS
Aspartic acid	MS
17-19	
Palmitic acid	MS
Stearic acid	MS
4-Ethyltetradecane	MS
2-Methylhexadecane	MS
Pentadecane	MS
20-22	
1-Methyl-α mannopyranoside	MS
Lactic acid	MS
Xylose	MS
23-27	
Phosphate[b]	GC, MS
Erythritol	MS
Erythrono-1,4-lactone	MS
32-39	
Glycolic acid	MS
3-Hydroxybutyric acid	MS
Gluconic acid	MS
Threitol	MS
Ribino-1,4-lactone	MS
Phosphate[b]	GC, MS
40-51	
3-Hydroxyisovaleric acid	MS
Phosphate[b]	GC, MS
Lactic acid	GC, MS
52-60	
Galactose	MS
Phosphate[b]	GC, MS
61-62	
Ethylene glycol	MS
Phosphate[b]	GC, MS
126-150[c]	
Benzoic acid	MS
Phosphate[b]	GC, MS
Lactic acid	MS
2,4-Dimethylhexane	MS
4-Methyl-2-propyl-1-pentanol	MS
2,2,3,3-Tetramethylhexane	MS
176-200	
Phosphate[b]	GC, MS
Decane	MS
3,4-Dimethylhexane	MS
Diethylphthalate	MS
Diisobutylphthalate	MS

[a] GC = gas chromatographic retention time, MS = mass spectra comparison.

[b] Analyzed as the trimethylsilyl (TMS) derivative of phosphate, tris(TMS)PO_4. Phosphate was identified in several chromatographic fractions in most effluents analyzed. It probably represents phosphate hydrolyzed from a phosphorylated organic by the trimethysilylation reagent.

[c] Mutagenic fraction.

Ten constituents were identified in the concentrate of the nitrified, chlorinated MMSE effluent (Table 29). Other than phosphate, phenylpyruvic oxime was the only compound tentatively identified in the mutagenic HPLC fractions.

Upper Thompson Sanitation District (EPSE)

Three sets of samples were collected at the Upper Thompson Sanitation District, Estes Park, Colorado on 10/19/77, 7/8/78, and 8/6/79. They were designated sets 1, 2, and 3 respectively. Each set consisted of an undisinfected (control) effluent and an ozonated effluent.

Set 1--

Mutagenicity--No mutagenic activity was detected in the 1000-fold concentrates of both the undisinfected (control) and ozonated sample (Table 11). In the initial phases of this research program, the criterion for bioscreening HPLC fractions was mutagenicity in the concentrated effluent. Therefore, the HPLC fractions from chromatographic separations of these concentrates were not tested for mutagenicity. As the program developed, it was concluded that this simple criterion for determining further characterization of effluent concentrates was not valid because mutagenic activity was detected in some HPLC fractions separated from effluent concentrates that had tested negative.

Chemical characterization--The concentrates of the undisinfected and ozonated effluents were chromatographed by preparative-scale HPLC. Due to a series of malfunctions in the GC/MS instrumentation, only limited chemical characterization data was obtained.

Set 2--

Mutagenicity--No mutagenic activity was detected in the 2000-fold concentrates of both the undisinfected (control) and ozonated EPSE sample (Table 11).

Preparative-scale chromatography of concentrate of the EPSE unozonated effluent produced 337 HPLC fractions, which were combined into 33 pooled fractions (Figure 52). They were tested for mutagenicity in both TA-1535 and TA-1538 with appropriate positive and negative controls. There were two positive and six slightly positive results in TA-1535 and five slightly positive results in TA-1538. These mutagenic data are given in Table 30.

HPLC chromatography of the concentrate of the EPSE ozonated effluent produced 300 HPLC fractions, which were combined into 38 pooled fractions (Figure 53). They were tested in strains TA-1535 and TA-1538. There were three positive samples in TA-1535 and five

TABLE 29. IDENTIFIED CONSTITUENTS IN HPLC FRACTIONS OF MMSE CHLORINATED, NITRIFIED EFFLUENT (9/19/79)

Constituent	LC fraction	Identification method[a]
Erythritol	12-14[b]	MS
Glucose	15-17	MS
Phosphate[c]	25-31	GC, MS
Rhamnose	25-31	MS
Lactic acid	32-34	MS
Phosphate[c]	35-39	GC, MS
Lactic acid	35-39	MS
1,3-Propanediol	35-39	MS
Xylose	35-39	MS
Phosphate[c]	40-47[b]	GC, MS
Phosphate[c]	52-56[b]	GC, MS
Phosphate[c]	66-70[b]	GC, MS
Phenylpyruvic oxime	66-70[b]	MS
Phenol	71-91	MS
1,2,3-Trimethoxypentane	71-91	MS
Phosphate[c]	71-91	GC, MS
Phosphate[c]	91-100	GC, MS
Phosphate[c]	101-125	GC, MS
Phosphate[c]	126-150	GC, MS
Phosphate[c]	151-175[b]	GC, MS
Phosphate[c]	201-225[b]	GC, MS
Phosphate[c]	251-275[b]	GC, MS

[a] GC = gas chromatographic retention, MS = mass spectra comparison.

[b] Mutagenic fraction.

[c] Analyzed as the trimethylsilyl (TMS) derivative of phosphate, tris(TMS)PO_4. Phosphate was identified in several chromatographic fractions in most effluents analyzed. It probably represents phosphate hydrolyzed from a phosphorylated organic by the trimethysilylation reagent.

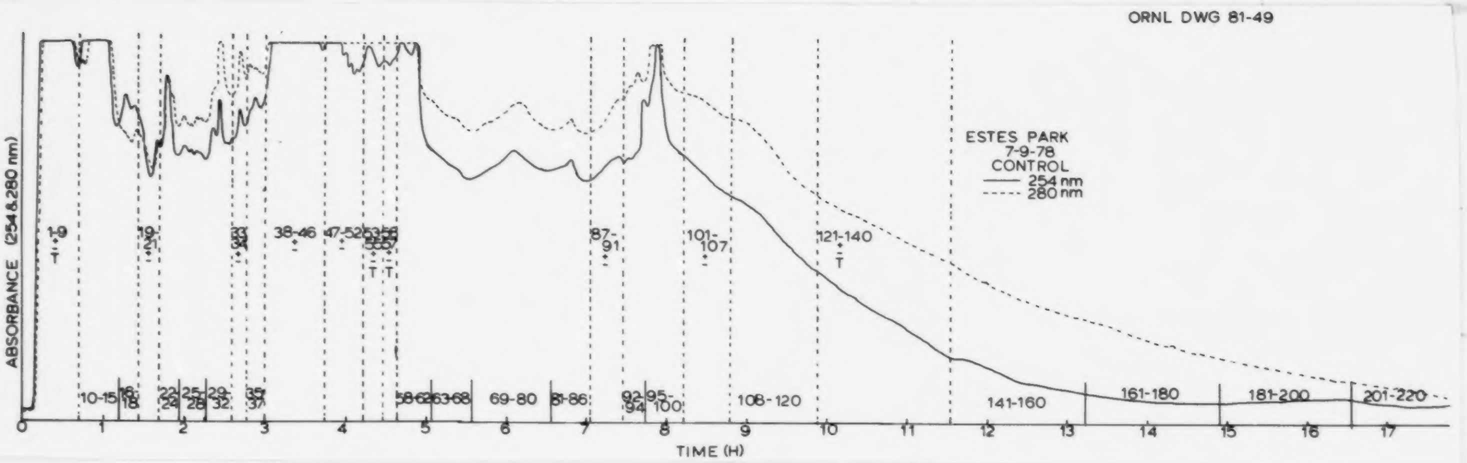

Figure 52. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the unozonated EPSE effluent (7/9/78). The mutagenic fractions are shown between dotted lines. The symbol + means positive mutagenic activity, ± means weakly positive, and T means toxic. Fractions 28 to 33 are not shown.

TABLE 30. MUTAGENIC DATA FOR HPLC FRACTIONS, EPSE

Effluent	ID	HPLC fraction no.	Mutagenic activity	TA-1535		TA-1538	
				Negative control, revertants	Test,[a] revertants	Negative control, revertants	Test,[a] revertants
Set 2							
Control (7/9/78)							
	1	1-9	±	17	36, 31		
	4	19-21	±	17	36, 35		
	8	33-34	±	17	26, 24		
	10	38-46	±			14	19, 32
	11	47-52	±	17	18, 36		
	12	53-55	±	17	34, 28	14	17, 28
	13	56-57	+	17	264, 18, T		
	18	87-91	±	26	29, 61	12	17, 24
	21	101-107	±			12	20, 34
	23	121-140	+	26	160, 14, T	12	28, 25
Ozonated (7/9/78)							
	2	11-12	±	26	53, 23, T		
	6	21-23	±	26	99, 13, T		
	7	24-28	+			17	39, 126
	8	29-33	±	26	26, 62	17	17, 52
	9	34-36	+	26	63, 113	17	45, 90
	10	37-40	±	26	13, 58		
	13	50-52	+			16	132, 261
	14[b]	53-55	+	26	~1000, 4000	16	~1000, 5000
	15[b]	56-57	+	26	~1000, 4000	16	~1000, 5000
	18	64-65	±	26	42, 58		
	22	77-83	±	26	27, 23, T		

TABLE 30. (continued)

Effluent	ID	HPLC fraction no.	Mutagenic activity	TA-1535		TA-1538	
				Negative control, revertants	Test,[a] revertants	Negative control, revertants	Test,[a] revertants
	25	95-99	±			16	21, 40
	30	120-125	±			16	35, 25, T
	31	126-150	±			16	33, 29, T
	32	151-164	±			16	47, 40, T
	35	201-225	±			16	18, 32
	36	226-245	±			16	25, 33
	37	246-270	±			16	41, 30
Set 3							
Control (8/16/79)							
	5	16-17	±	30	48, 41, T		
	20	63-71	±			14	13, 29
Ozonated (8/16/79)							
	1	1-9	±	18	12, 50	16	34, 61
	7	27-33	+	18	2000, 2500	16	1000, 2000
	9	39-40	±			16	28, 36
	11	44-45	±			16	22, 23
	15	59-62	±			16	37, 39

[a] First number = revertants/50 μL, second number = revertants/150 μL, T = toxic.

[b] Probably histidine or contamination.

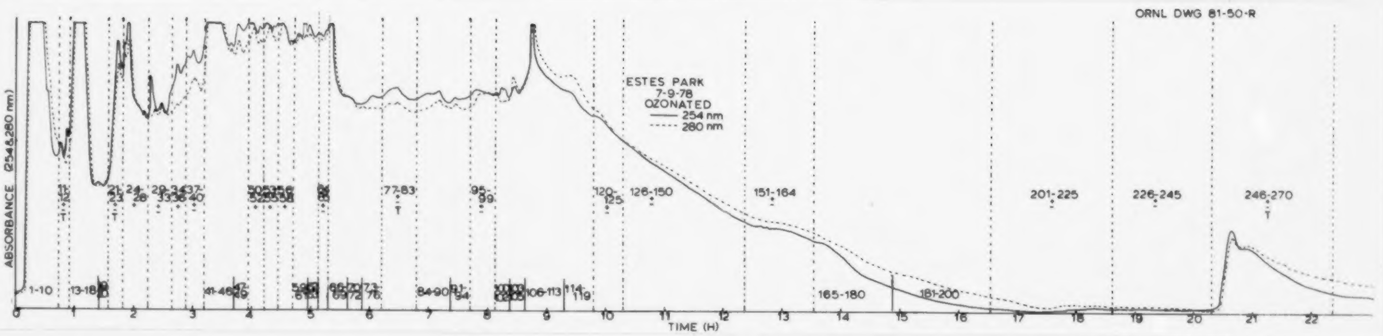

Figure 53. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the ozonated EPSE effluent (7/9/78). The mutagenic fractions are shown between dotted lines. The symbol + means positive mutagenic activity, ± means weakly positive, and T means toxic. Fractions 37 and 38 are not shown.

positives in TA-1538. In addition TA-1535 showed six slightly positive and TA-1538 showed eight slightly positive. These mutagenic data are given in Table 30.

Histidine was tentatively identified in two of the pooled fractions (53-55, 56-57) that gave strongly positive tests in both TA-1535 and TA-1538. Because the histidine requiring test bacteria would grow rapidly in the presence of histidine, these test results were false positives. That is, the positive results were not due to mutagenic activity in the pooled fractions. The same highly positive results were not obtained with the same pooled HPLC fractions for the unozonated effluent. Thus, it is possible that ozonation released peptides in the ozonated effluent and the peptides acted as histidine surrogates for the test bacteria.

Chemical characterization--The HPLC fractions presumed to contain histidine were examined by GC and LC. The retention times of a histidine standard matched those of a similarly analyzed suspect peak derived from fractions 53-55 and 56-57 of the ozonated effluent. Its concentration was estimated at one microgram-per-liter in the effluent. A standard mass spectrum of histidine is not available in the literature apparently because of its instability, and efforts at obtaining a mass spectrum of the more stable trimethylsilyl derivative were not successful.

Compounds identified in the control and ozonated effluent concentrates are listed in Table 31 and 32, respectively.

The organic content for the effluent samples in this collection period seemed atypically high (see Appendix). This effect was attributed to development of a high concentration of algae, which apparently reduced the disinfection effectiveness of the ozone. What effect this phenomenon had on the mutagenicity and chemical constituents of the effluents is not known.

Set 3--

Mutagenicity--No mutagenic acitivity was detected in the 2000-fold concentrates of both the undisinfected (control) and ozonated EPSE effluents (Table 12). Subsequent preparative HPLC chromatography of the concentrate of the unozonated effluent produced 300 eluate fractions that were combined into 34 pooled fractions for mutagenicity testing as shown in Figure 54. Only two of these were slightly mutagenic (Table 30).

Chromatography of the concentrate of the EPSE ozonated effluent produced 300 eluate fractions, which were combined into 27 pooled fractions for mutagenicity testing (Figure 55). Of these samples, one was slightly positive in TA-1535, four were slightly positive in TA-1538, and one was positive in both strains. Previous testing of

TABLE 31. IDENTIFIED CONSTITUENTS IN HPLC FRACTIONS OF UNOZONATED (CONTROL) SECONDARY EFFLUENT (7/9/81)

Constituent	LC fraction	Identification method[a]
Phosphate	56-57	GC, MS
Tyrosine	56-57	GC, LC
Cystine	56-57	GC
5-Hydroxyuridine	56-57	GC
Phosphate	56-57	GC/MS

[a] GC = gas chromatographic retention time, MS = mass spectra, LC = liquid chromatographic elution position.

TABLE 32. IDENTIFIED CONSTITUENTS IN HPLC FRACTIONS OF OZONATED SECONDARY EFFLUENT (7/9/78)

Constituent	LC fraction	Identification method[a]
Phosphate	53-53	GC, MS
Ketoglutaric acid	53-55	GC
Histidine	53-55	GC, LC
Phosphate	56-58	GC, LC
Phenylacetamide	56-58	GC, LC
Phenylpuruvic acid	56-58	GC
Histidine	56-58	GC, LC
Homocystine	56-58	GC
Palmitic acid	56-58	GC, MC
Stearic acid	56-58	MS

[a] GC = gas chromatographic retention time, MS = mass spectra, LC = liquid chromatographic elution position.

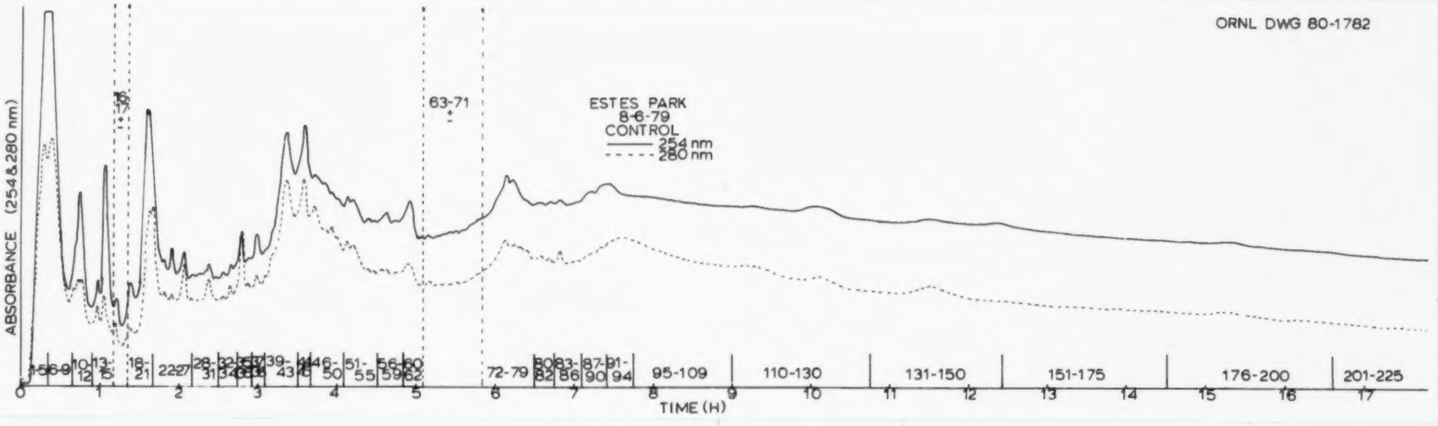

Figure 54. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the unozonated EPSE effluent (8/6/79). The mutagenic fractions are shown between dotted lines. The symbol ± means weakly positive mutagenic activity. Fractions 30 to 34 are not shown.

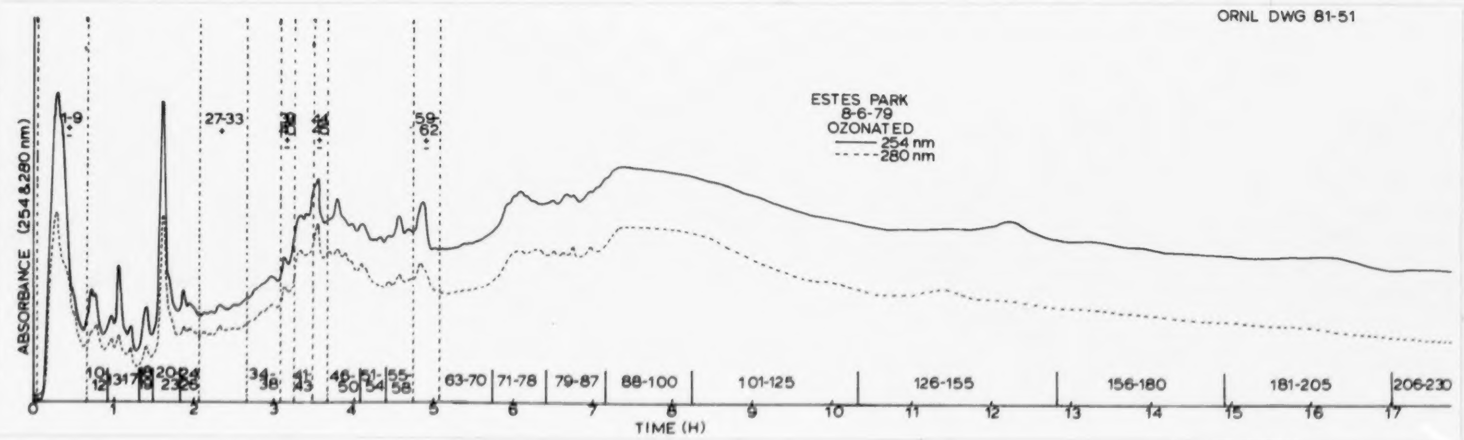

Figure 55. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the ozonated EPSE effluent (8/6/79). The mutagenic fractions are shown between dotted lines. The symbol + means positive mutagenic activity and ± means weakly positive. Fractions 25 to 27 are not shown.

chromatographic fractions showed much sensitivity to both strains. The assumed positives in mutagenic testing for set 2 rarely showed toxicity of mutagenicity for similar HPLC fractions in the set 3 test series.

These mutagenicity results are considerably different from the results obtained for Set 2 which showed either much mutagenicity or "false positives" possibly due to contamination or other artifacts. Set 3 mutagenicity testing utilized a modified bacterial testing procedure that incorporated filtration and very strict quality control for bacterial tester strains. The results for the set 3 test series should be an accurate appraisal of the mutagenicity in the EPSE control effluent.

For set 3, it was concluded that ozonation destroyed some mutagenic components (e.g., those in fractions 16-17 and 63-71, Figure 54) and produced other mutagenic constituents (e.g., the mutagenic fractions shown in Figure 55).

Chemical characterization--Nine constituents were identified in the concentrate of the control effluent (Table 33). No compounds were identified in mutagenic HPLC fractions.

Twenty-five constituents were identified in the concentrate of the ozonated effluent (Table 34). All of the identified compounds were in mutagenic fractions but none were known to be mutagens.

The very positive mutagenic tests of HPLC fractions 27-33 provide presumptive evidence that histidine may be present. The apparent presence of histidine in the ozonated effluent was also observed in the set 2 ozonated effluent (7/8/78). The lack of histidine in the control effluent for both set 2 and 3 indicates that ozonation may be a causal factor for the presence of histidine in the ozonated effluent. This interesting phenomenon needs further investigation.

As was the case in set 2, the sample collection period coincided with plant operating conditions that resulted in higher than normal organic loadings (high BODs in the ozonated effluent) and limited disinfection (see Appendix).

Taft Center Pilot Wastewater Treatment Plant (CSE)

Two sets of samples were collected at the Taft Center Pilot Wastewater Treatment Plant, Cincinnati, Ohio on 9/12/77 and on 5/24/78. Each set consisted of undisinfected (control) and ozonated effluents. The two sets of samples were concentrated and examined.

Set 1--

Mutagenicity--The 1500- to 1600-fold concentrates of the undisinfected (control) effluent and ozonated effluent were extensively

TABLE 33. IDENTIFIED CONSTITUENTS IN HPLC FRACTIONS OF UNDISINFECTED (CONTROL) EFFLUENT (8/6/79)

HPLC fraction/constituent	Identification method[a]
18-21	
Benzoic acid	MS
Phosphate[b]	GC, MS
Glucitol	MS
22-24	
Dichlorobenzoic acid	MS
Palmitic acid	MS
Glycerol	MS
Phospohate[b]	GC, MS
26-27	
O-Decylhydroxylamine	MS
28-31	
Propylene glycol	MS
Phosphate[b]	GC, MS
50-59	
Glycerol	MS
Phosphate[b]	GC, MS
95-109	
Phosphate[b]	GC, MS
110-130	
Phosphate[b]	GC, MS
Xylitol	MS

[a] MS = mass spectra comparison, GC = gas chromatographic retention time.

[b] Analyzed as the trimethylsilyl (TMS) derivative of phosphate, tris(TMS)PO_4. Phosphate was identified in several chromatographic fractions in most effluents analyzed. It probably represents phosphate hydrolyzed from a phosphorylated organic by the trimethysilylation reagent.

TABLE 34. IDENTIFIED CONSTITUENTS IN MUTAGENIC HPLC FRACTIONS OF EPSE OZONATED EFFLUENT (8/6/79)

HPLC fraction/constituent[a]
10-12
2,4-Dimethyl-2,4-disilapentane
2-Aminoethanol
Phosphate[b]
2-Methyl-1,3,6-trioxocane
Threono-1,4-lactone
Xylono-1,4-lactone
Xylose
13-17
Phthalic acid
Lactic acid
2-Hydroxyisobutyric acid
Phosphate[b]
20-23
Urea
Glycerine
Glycine
Phosphate[b]
Dichlorobenzoic acid
Benzoic acid
Palmitic acid
Stearic acid
Pentacosane
Methyleicosane
27-33
Histidine[c]
Ethylene glycol
3,6,9-Trioxa-2-silaundecane-2-3-dimethyl
3,7-Dioxa-2,8-disilanonane-2-one-2,2,8,8-tetramethyl
Threitol
Erythritol
Xylose
Phosphate[b]
44-45
Phosphate[b]
591-62
Phosphate[b]
101-125
Propanoic acid
Phosphate[b]

[a] All constituents identified by GC/MS.

[b] Analyzed as the trimethylsilyl (TMS) derivative of phosphate, tris(TMS)PO_4. Phosphate was identified in several chromatographic fractions in most effluents analyzed. It probably represents phosphate hydrolyzed from a phosphorylated organic by the trimethysilylation reagent.

[c] Possible identification. Identified by GC retention times and "positive" mutagenicity to both TA-1535 and TA-1538.

tested for mutagenicity, but all tests were negative (Table 11). No bacterial mutagenicity tests were conducted on the HPLC fractions because in the initial phases of this research program the criterion for further testing was mutagenic activity in the concentrate.

Chemical characterization--The chromatograms of uv-absorbing constituents separated by preparative-scale HPLC from the concentrates of the control and ozonated CSE effluents are given in Figures 56 and 57. An obvious conclusion from comparison of the two chromatograms is that ozonation destroyed many constituents that eluted in the 2 to 15 hr range.

Thymine, uracil and isovaleric acid were identified in the unozonated control sample but were not detected in the ozonated sample. Phosphate and mass spectral fragments indicative of a nucleotide were detected in the unozonated sample, and phosphate was also identified in the ozonated sample.

Set 2--

Mutagenicity--No mutagenic activity was detected in the 2000-fold concentrates of both the undisinfected (control) and ozonated CSE effluents (Table 11).

Preparative-scale HPLC chromatography of the concentrate of the control effluent produced 245 eluate fractions, which were combined into 24 pooled fractions for bacterial mutagenicity testing (Figure 58). Two of the fractions were slightly positive in TA-1535, and all were negative in TA-1538 (Table 35). In this case the total mutagenicity increased with HPLC fractionation because the concentrate was tested as negative.

The preparative-scale chromatography of the concentrate of the ozonated effluent produce 238 eluate fractions, which were combined into 22 pooled fractions (Figure 59). Two fractions were found to be slightly mutagenic in TA-1535, which detects base pair substitution mutagens (Table 35). These slightly positive fractions were different than those slightly positive in the control effluent. The mutagenicity results corroborate previous conclusions that ozonation may destroy some mutagenic constituents and produce others.

Chemical characterization--Only phosphate was identified in the mutagenic fractions of the CSE control effluent. In the ozonated effluent, phosphate was identified in both mutagenic fractions and 1,2,3-trimethylpentane was identified in fractions 49-60.

Meander Creek Wastewater Treatment Plant (MCSE)

Two sets of samples were collected at the Meander Creek Wastewater Treatment Plant, Mineral Ridge, Ohio, on 6/15/78 and 10/5/78. Each set consisted of a control (undisinfected) and an ozonated effluent.

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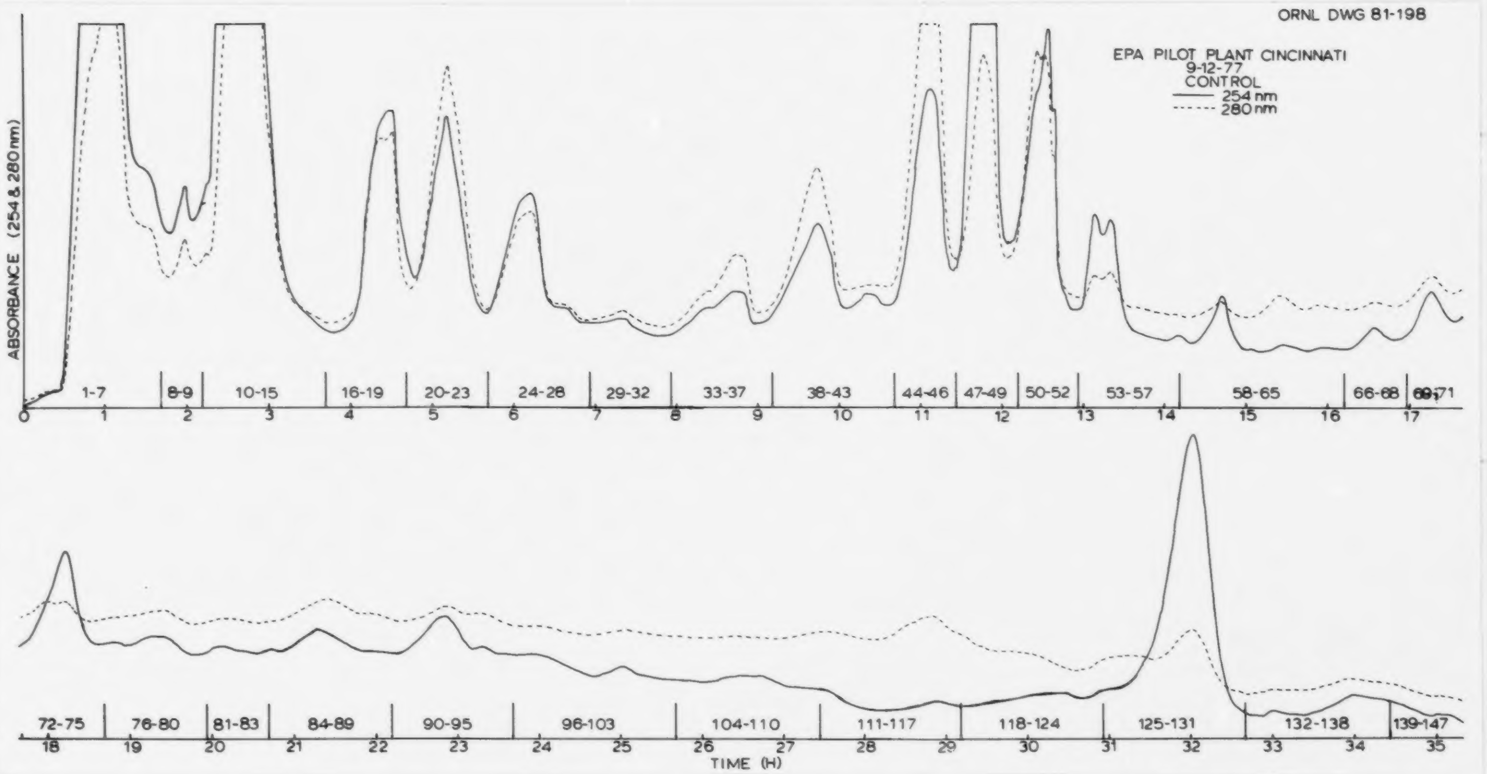

Figure 56. Preparative-scale HPLC chromatogram of uv-absorbing constituents for a 1500-fold concentrate of the undisinfected CSE effluent (9/12/77). Pooled eluate fractions for further testing are designated.

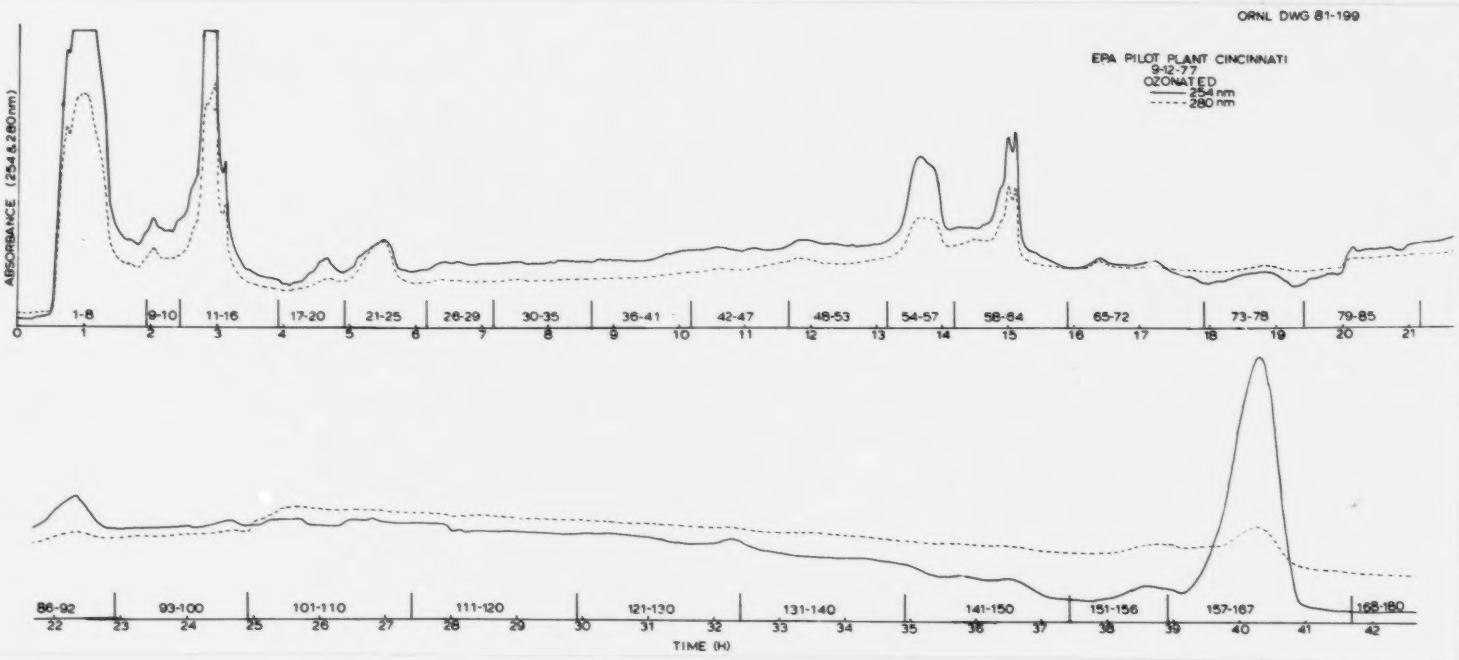

Figure 57. Preparative-scale HPLC chromatogram of uv-absorbing constituents for a 1600-fold concentrate of the ozonated CSE effluent (9/12/77). Pooled eluate fractions for further testing are designated.

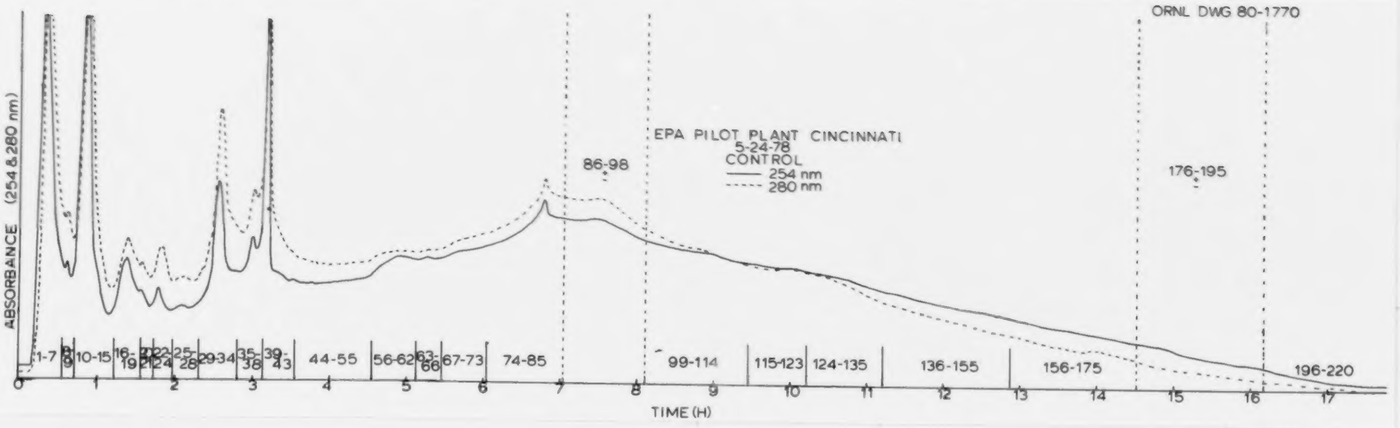

Figure 58. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the undisinfected (control) CSE effluent (5/24/78). The mutagenic fractions are shown between dotted lines. The symbol ± means weakly positive mutagenic activity. Fraction 24 is not shown.

TABLE 35. MUTAGENIC DATA FOR HPLC FRACTIONS, CSE

				TA-1535		TA-1538	
Effluent	ID	HPLC fraction no.	Mutagenic activity	Negative control, revertants	Test,[a] revertants	Negative control, revertants	Test,[a] revertants
Control (5/24/78)							
	16	86-98	±	29	55, 104		
	22	176-195	±	29	60, 126		
Ozonated (5/24/78)							
	8	49-60	±	30	86, 38, T		
	9	61-70	±	30	92, 78, T		

[a] First number = revertants/50 μL, second number = revertants/150 μL, T = toxic.

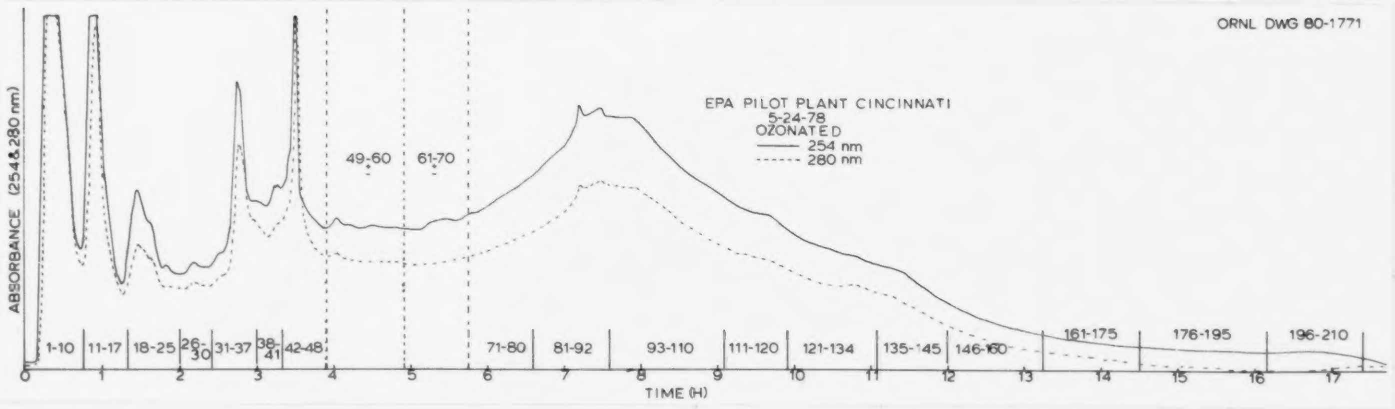

Figure 59. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the ozonated CSE effluent (5/24/78). The mutagenic fractions are shown between dotted lines. The symbol ± means weakly positive mutagenic activity.

Set 1--

Mutagenicity--The 2000-fold concentrates of the MCSE control and ozonated effluents were tested for mutagenicity. All tests were negative (Table 11). Preparative-scale HPLC chromatography of the concentrate of the control effluent produced 220 eluate fractions which were combined into 19 pooled fractions (Figure 60). These fractions were tested for mutagenicity in both TA-1535 and TA-1538. One fraction was slightly positive in both strains (Table 36).

Corresponding chromatography of the concentrate of the ozonated effluent produced 210 eluate fractions, which were combined into 18 pooled fractions (Figure 61). The pooled samples were not tested for mutagenicity.

Chemical characterization--No chemical analyses were made on the effluent samples.

Set 2--

Mutagenicity--The approximate 3000-fold concentrates of the control and ozonated MCSE effluents were tested. The control concentrate was negative and the ozonated concentrate was slightly positive in TA-1538 (Table 11). Preparative-scale HPLC chromatography of the concentrate of the control effluent produced 300 eluate fractions, which were combined into 28 pooled fractions (Figure 62). One fraction was positive and five were slightly positive in TA-1535; one was slightly positive in TA-1538 (Table 36).

Corresponding chromatography of the concentrate of the control effluent produced 300 eluate fractions, which were combined into 27 pooled fractions (Figure 63). Of these, 19 were positive in both strains (Table 36). This result was reason to suspect that the samples were contaminated with other bacterial strains. The samples were rerun, but the tests were still inconclusive due to possible reverting of the Salmonella strains to wild type, inconsistency of samples, or contamination. Consequently, another preparative-scale HPLC run was made with the concentrate of the MCSE ozonated effluent. The HPLC fractions were combined into 27 samples. The fractionated samples continued to show a very high frequency of positive tests in both strains, indicating a continuing problem. Sixteen of the 27 samples were equally positive in both TA-1535 and TA-1538.

As a result of the "false positive" mutagenic test and several other apparently anomalous results discussed in previous sections, this problem was investigated thoroughly, as will be discussed in a later section. The major conclusion from that study was that the tester strains had reverted to wild type despite quality control procedures.

Chemical characterization--Fourteen constituents were identified in mutagenic fractions of the control MCSE effluent (Table 37).

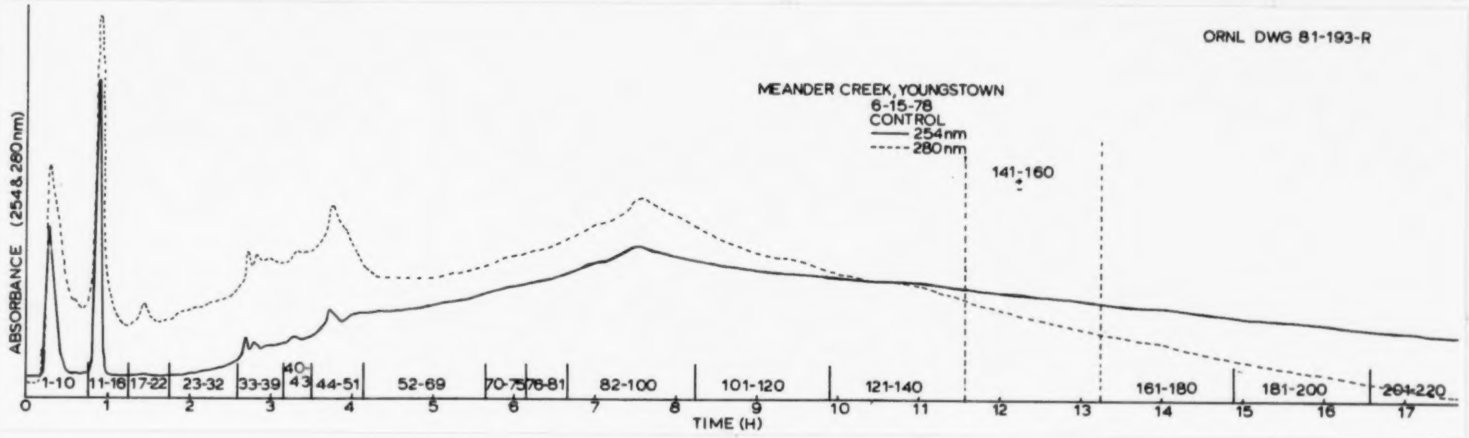

Figure 60. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the undisinfected (control) MCSE effluent (6/15/78). Fractions 141-160 tested weakly positive when tested with TA-1535, a bacterial tester strain sensitive to base pair substitution mutagens. Fractions 18 and 19 are not shown.

TABLE 36. MUTAGENIC DATA FOR HPLC FRACTIONS, MCSE

				TA-1535		TA-1538	
Effluent	ID	HPLC fraction no.	Mutagenic activity	Negative control, revertants	Test,[a] revertants	Negative control, revertants	Test,[a] revertants
Control (6/15/78)							
	15	141-160	±	23	35, 76	18	56, 78
Ozonated (6/15/78)							
	No data						
Control (10/15/78)							
	6	22-26	±	24	39, 72		
	7	27-30	±	24	38, 65		
	8	31-34	±	24	21, 89		
	13	47-48	±	24	37, 95		
	14	49-51	±	24	52, 27, T		
	20	116-140	±	30	50, 420		
	28	281-300	±			10	16, 30
Ozonated (10/5/78)							
	Data unacceptable						

[a] First number = revertants/50 µL, second number = revertants/150 µL, T = toxic.

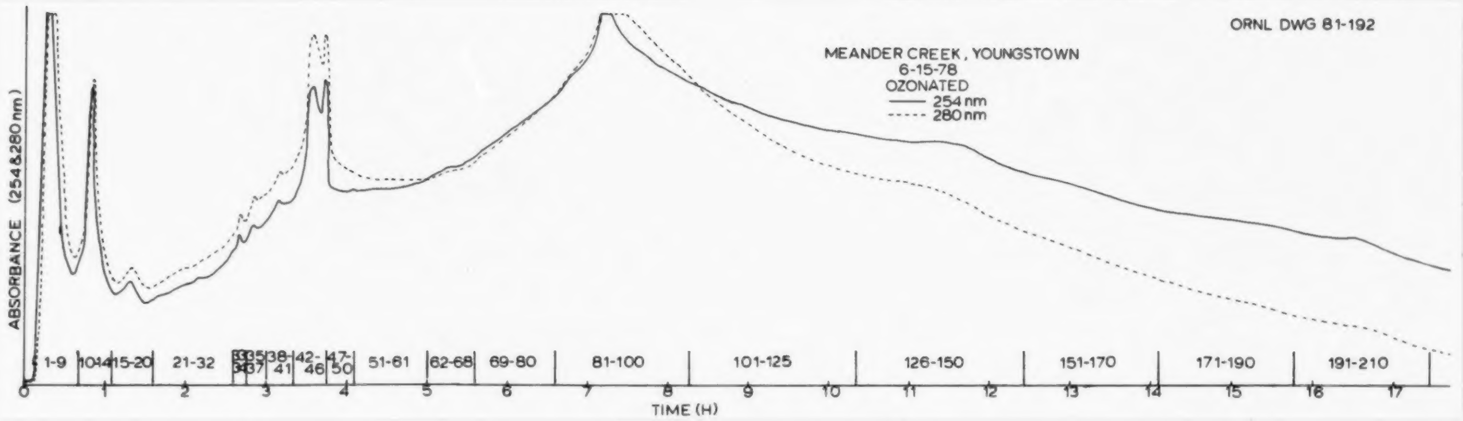

Figure 61. Chromatogram of uv-absorbing constituents in a 2000-fold concentrate of the ozonated MCSE effluent (6/15/78).

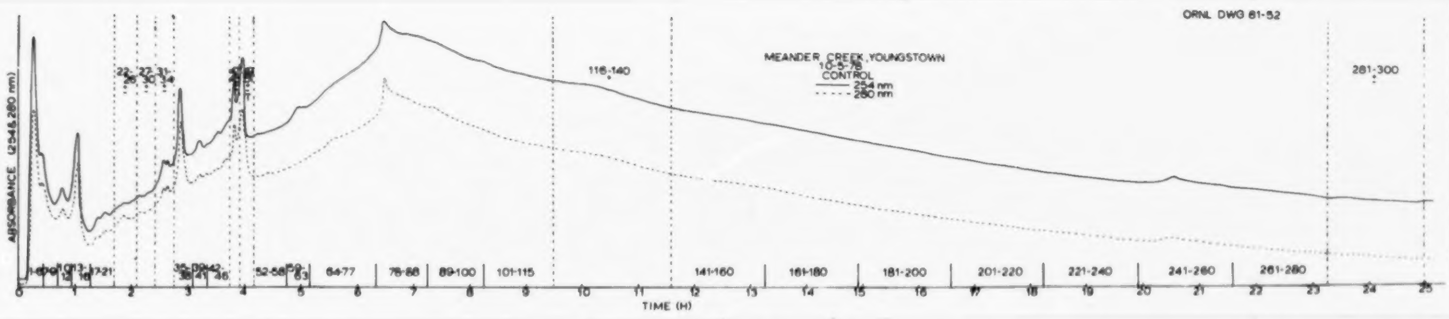

Figure 62. Chromatogram of uv-absorbing constituents in a 3000-fold concentrate of the undisinfected (control) MCSE effluent (10/5/78). The mutagenic fractions are shown between dotted lines. The symbol + means positive mutagenic activity, ± means weakly positive, and T means toxic.

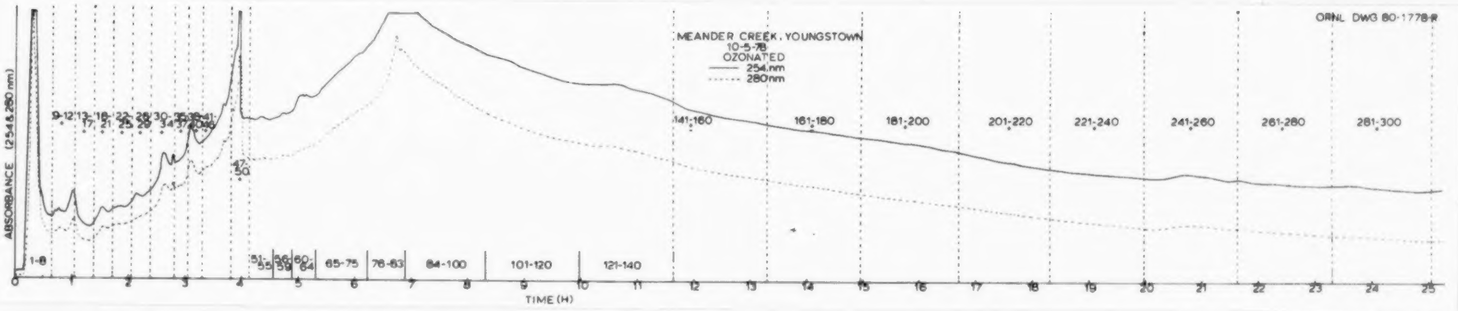

Figure 63. Chromatogram of uv-absorbing constituents in a 3000-fold concentrate of a ozonated MCSE effluent (10/5/78).

TABLE 37. IDENTIFIED CONSTITUENTS IN MUTAGENIC HPLC FRACTIONS OF UNDISINFECTED (CONTROL) MCSE EFFLUENT (10/5/78)

HPLC fraction/constituent[a]
22-26 Lactic acid Phosphate[b] Palmitic acid
27-30 1-Methylmannofuranoside Phosphate[b] Stearic acid
31-34 Phosphate[b]
47-48 Benzoic acid Cyclobutanol *N*,*N*'-diethyl-*N*,*N*'-dimethyl-*p*-phenylenediamine Phosphate[b]
49-51 Nordodecane Phosphate[b] Urea
116-140 Erythrose Erythritol Lactic acid Phosphate[b]
261-280 2,5-Dimethyl-3,4-hexanediol Phospohate[b]
281-300 Glycerine Lactic acid Phosphate[b]

[a] All identifications were made by GC/MS.

[b] Analyzed as the trimethylsilyl (TMS) derivative of phosphate, tris (TMS) PO_4. Phosphate was identified in several chromatographic fractions in most effluents analyzed. It probably represents phosphate hydrolyzed from a phosphorylated organic by the trimethysilylation reagent.

Again, the same general distribution for phosphate, benzoic acid, lactic acid, and fatty acids was observed. The identification of the aromatic amine in fractions 47-48 is interesting and may represent a possible mutagen.

The fractions from the HPLC separation of the ozonated MCSE effluent were not characterized.

Northwest Bergen County Wastewater Treatment Plant (NBCSE)

Two sets of samples were collected at the Northwest Bergen County Wastewater Treatment Plant, Waldwick, New Jersey: Set 1, undisinfected (control) and ultraviolet-light-irradiated effluents, collected on 4/21/78, and Set 2, undisinfected (control), ultraviolet-light-irradiated, and chlorinated effluents, collected on 8/28/78.

Set 1--

Mutagenicity--The approximate 3000-fold concentrates of the NBCSE control and uv-irradiated effluents were tested for mutagenicity using both TA-1535 and TA-1538 strains. All tests were negative (Table 11). No bacterial mutagenicity tests were conducted on the HPLC fractions.

Chemical characterization--HPLC chromatograms of the uv-absorbing constituents separated from the concentrates of the control and uv-irradiated effluents are given in Figures 64 and 65, respectively. The large uv-absorbing peak that eluted at 11.5 hr in Figure 65 was apparently produced by uv irradiation. Identified constituents in this chromatographic peak were oxalic acid, stearic acid, o-hydroxyphenylacetic acid, glycerine, phosphate, and 2,2,3,3-tetramethylhexane. It is thought that the presence of several smaller molecular moieties, (e.g., oxalic acid, glycerine, phosphate, etc.) may represent breakdown products from a larger molecular complex.

The analytical-scale HPLC chromatogram of uv-absorbing constituents in the control and uv-irradiated effluents are compared in Figure 66. The two superimposed chromatograms are remarkably similar except for the destruction of a peak at 14 hr in the control and the formation of a peak at 9 hr in the uv-irradiated effluent. This high degree of similarity between the two chromatograms corroborates the conclusion that chemical effects of uv-irradiation on nonvolatile organics appear to be minimal.

Set 2--

Mutagenicity--The approximate 1000-fold concentrate of the control effluent tested negative; however, the uv-irradiated and chlorinated effluent concentrates were slightly mutagenic in TA-1535 (Table 11).

Preparative-scale HPLC chromatography of the concentrate of control effluent produced 250 eluate fractions, which were combined

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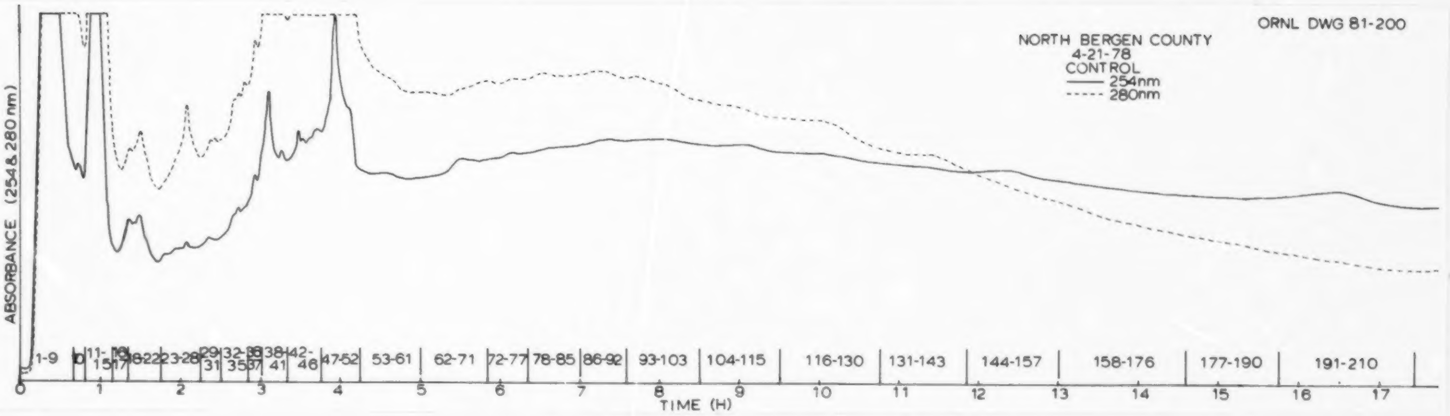

Figure 64. Preparative-scale HPLC chromatogram of uv-absorbing constituents in a 3000-fold concentrate of the NBCSE undisinfected (control) effluent (4/21/78).

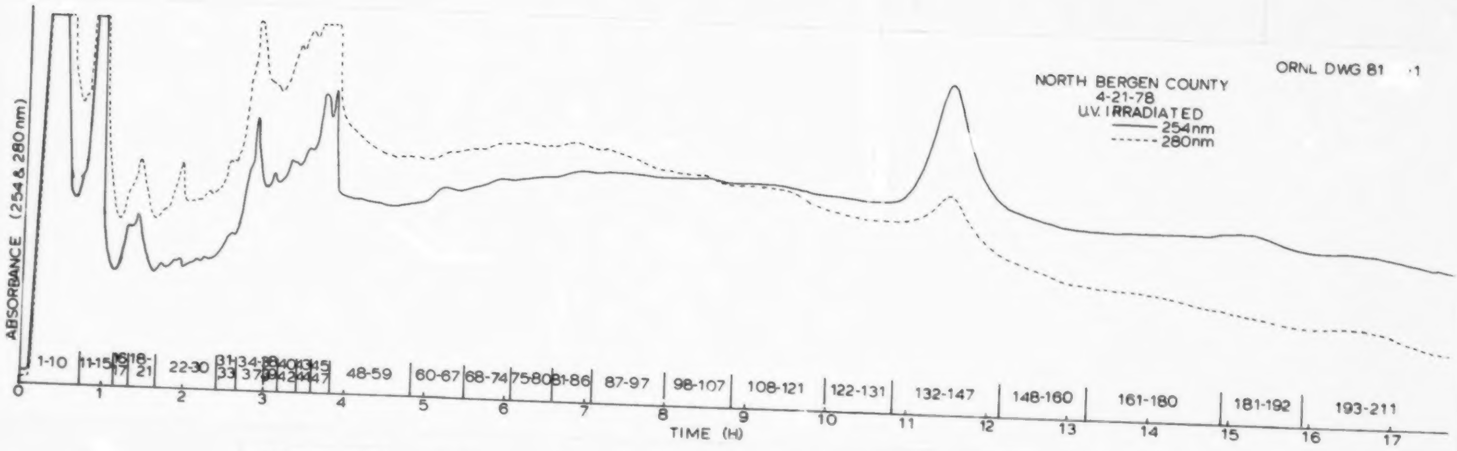

Figure 65. Preparative-scale HPLC chromatogram of uv-absorbing constituents in a 3000-fold concentrate of the NBCSE ultraviolet-light-irradiated effluent (4/21/78).

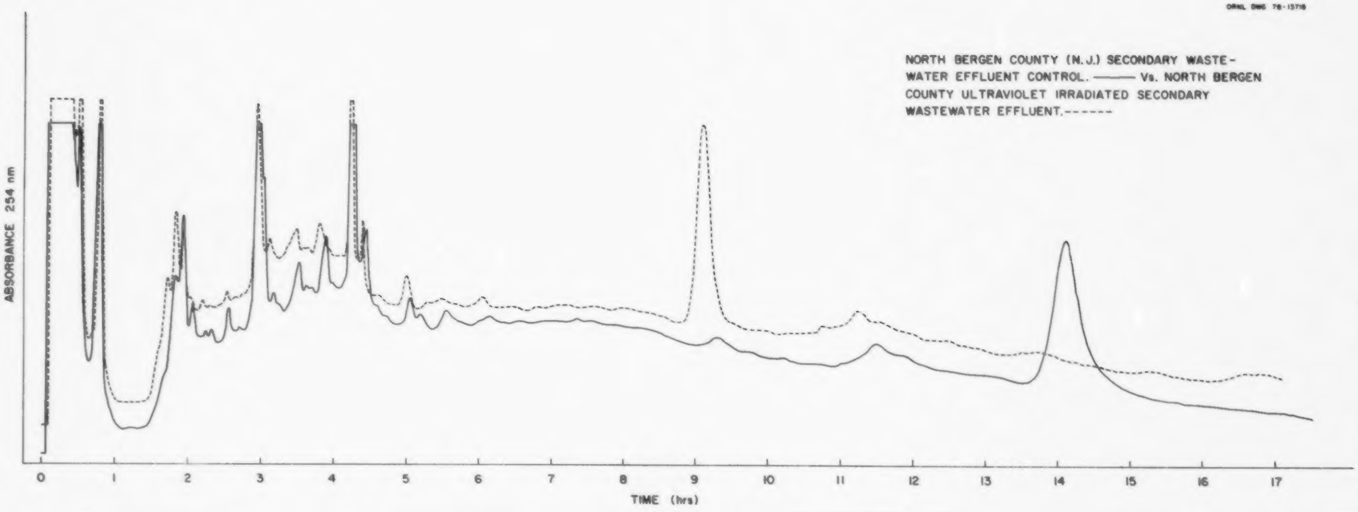

Figure 66. Analytical-scale HPLC chromatograms of the uv-absorbing constituents in a 3000-fold concentrate of the control and uv-irradiated NBCSE effluents (4/21/78).

into 18 pooled fractions (Figure 67). The samples were tested for mutagenicity in TA-1535 and TA-1538. There were three positive but highly toxic tests in TA-1535 and one slightly positive and toxic test in TA-1538 (Table 38). It is interesting to note that the control concentrate tested negative.

Two hundred and fifty HPLC eluate fractions resulting from the chromatography of the concentrate of the uv-irradiated NBCSE effluent were combined into 19 pooled fractions for mutagenicity testing in TA-1535 and TA-1538 (Figure 68). All tested negative (Table 38). The original concentrate from which the HPLC uv-irradiated fractions were derived tested slightly positive in both TA-1535 and TA-1538. This result indicates that some mutagenic activity may have been removed by HPLC chromatography.

Similarly, the HPLC eluate fractions from chromatography of the NBCSE chlorinated effluent were combined into 24 pooled fractions (Figure 69). All HPLC fractions were negative. The original concentrate tested slightly positive in TA-1535.

Apparently, uv irradiation and chlorination eliminated the chromatographic mutagenic components found in the control effluent. Thus, uv irradiation does chemically affect some chromatographic constituents. Chlorination destroyed the chromatographic mutagenic components in the control effluent also.

Chemical characterization--Four constituents were identified in the mutagenic HPLC fractions separated from the concentrate of the control effluent (Table 39). Again, a pattern of small molecular weight constituents was found in the analyzed fractions.

MUTAGENICITY TESTING PROBLEM

In the later phases of this research program, during a period of extensive mutagenicity testing of HPLC fractions, a large number of very positive test results began to appear, many in both TA-1535 and TA-1538. Consequently, the problem was thoroughly investigated to determine the cause of the anomalous tests.

Hypothetical Causes

It was reasoned that the very positive test results might be due to one or several of the following factors:

1. Actual mutagenicity of the sample.

2. Faulty mutagenic test bacterial strains or inadequate test procedure.

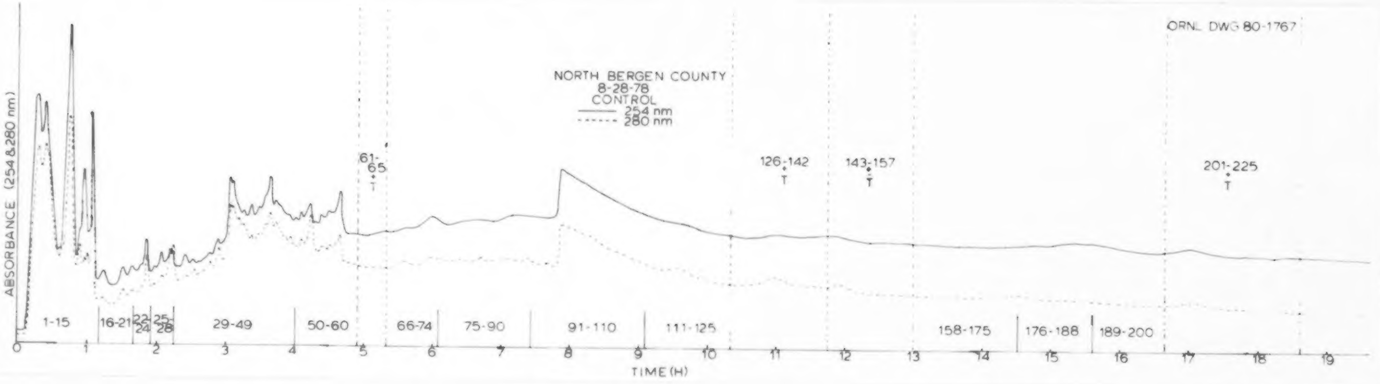

Figure 67. Chromatogram of uv-absorbing constituents in a 1000-fold concentrate of the control NBCSE effluent (8/28/78). Mutagenic fractions are shown between dotted lines. The symbol + means positive mutagenic activity, ± means weakly positive, and T means toxic. Fraction 18 is not shown.

TABLE 38. MUTAGENIC DATA FOR HPLC FRACTIONS, NBCSE

Effluent	ID	HPLC fraction no.	Mutagenic activity	TA-1535		TA-1538	
				Negative control, revertants	Test,[a] revertants	Negative control, revertants	Test,[a] revertants
Control (8/28/78)							
	7	61-65	±	33	243, 38, T		
	12	126-142	+	33	441, 86, T		
	13	143-157	±			13	44, 18, T
	17	201-225	+	33	479, 140, T		
Ultraviolet-light-irradiated (8/28/78)	All tests negative						
Chlorinated (8/28/78)	All tests negative						

[a] First number = revertants/50 μL, second number = revertants/150 μL, T = toxic.

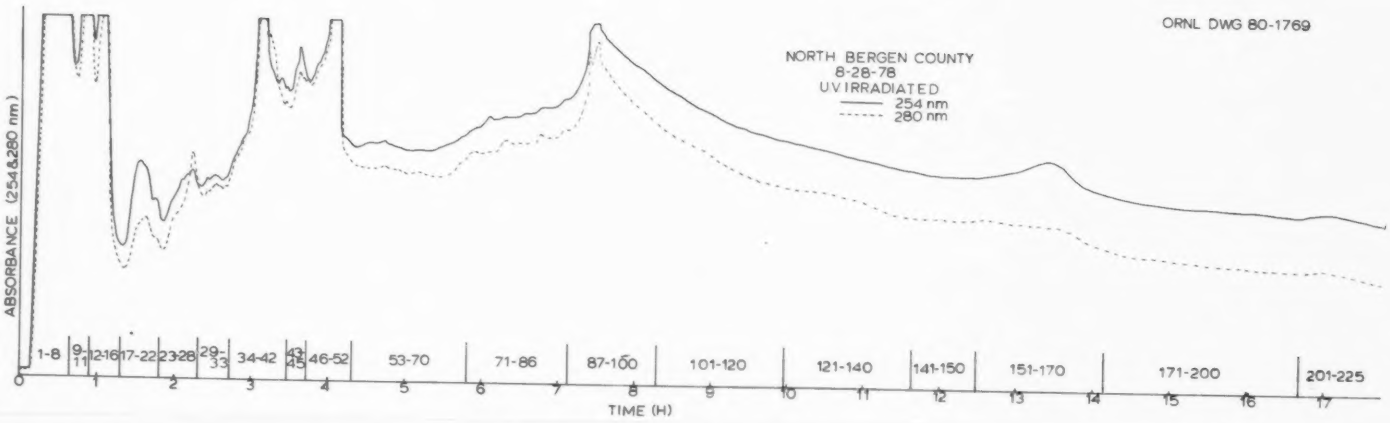

Figure 68. Chromatogram of uv-absorbing constituents in a 1000-fold concentrate of the uv-irradiated NBCSE effluent (8/28/78). No mutagenic fractions were found.

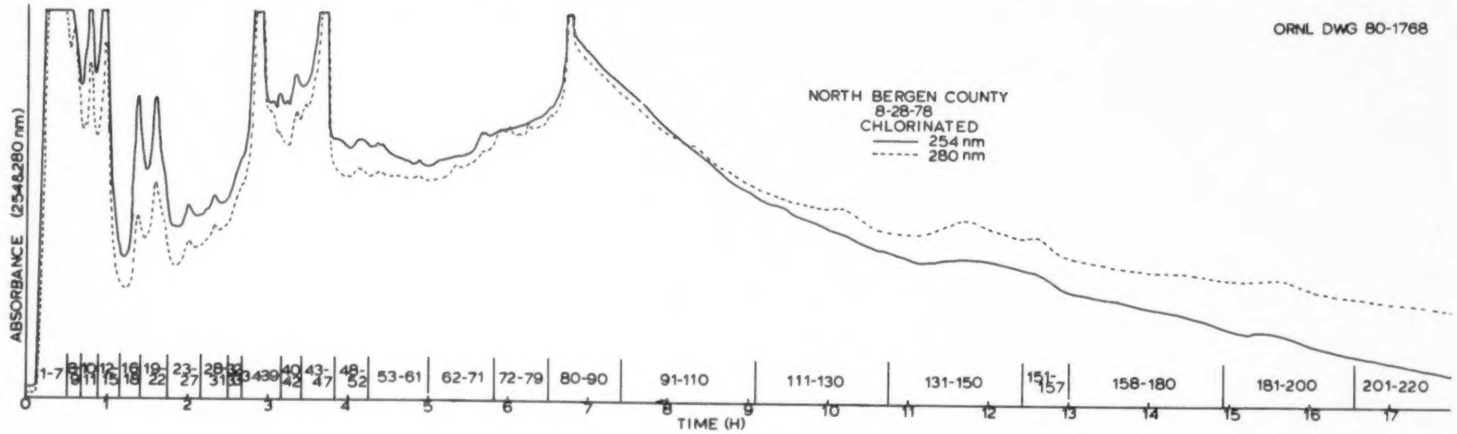

Figure 69. Chromatograms of uv-absorbing constituents in a 1000-fold concentrate of the chlorinated NBCSE effluent (8/28/78). No mutagenic fractions were found.

TABLE 39. IDENTIFIED CONSTITUENTS IN MUTAGENIC HPLC FRACTIONS OF UNDISINFECTED (CONTROL) NBCSE EFFLUENT (8/28/78)

HPLC fraction/constituent[a]	
61-65 Phenol Propanoic acid Phosphate[b]	143-157 Phosphate[b]
126-142 2-Pyridinecarboxylic acid Phosphate[b] Propanoic acid	201-225 Phosphate[b]

[a] All identifications were made by GC/MS.

[b] Analyzed as the trimethylsilyl (TMS) derivative of phosphate, tris(TMS)PO_4. Phosphate was identified in several chromatographic fractions in most effluents analyzed. It probably represents phosphate hydrolyzed from a phosphorylated organic by the trimethysilylation reagent.

3. Bacterial contamination.

   a. Bacterial contamination might have occurred during the mutagenicity test.

   b. Bacteria might have contaminated the HPLC resin, been eluted into the HPLC fractions, and survived the lyophilization preparation procedure.

   c. Bacterial contamination might have occurred during preparation of HPLC sample for test.

4. Chemical contamination with a mutagen or chemical that gave a false positive.

   a. Contamination might have resulted from buildup on HPLC resin and subsequent elution.

   b. Contamination might have resulted from a chemical being introduced in the preparation for mutagenicity test or during the test procedure.

It was decided to systematically explore these possibilities.

Hypothesis 1

It was unlikely that all the positive tests represented actual mutagenicity because each bacterial tester strain responded positively. A chemical mutagen will not usually be positive in all tester strains.

Hypothesis 2

Bacterial tester strains TA-98, TA-100, TA-1535, TA-1537, and TA-1538 differ from wild type (potentially contaminating) bacteria in several important ways. Each of the tester strains carries the uvrB mutation which renders them uv sensitive, whereas wild type bacteria are relatively uv resistant. Each also carries a mutation of the lipopolysaccharide coating (deep-rough character) making the cell membrane more permeable to potential mutagens. Two of the strains (TA-98 and TA-100) carry an R factor which renders them more sensitive to many mutagens.[35]

The bacterial tester strains used in these experiments were periodically tested for the possibility of changes that might lead to faulty experimental results. The spot tests used to check the bacterial strains included uv irradiation which tests for the presence of the uvrB mutation, crystal violet sensitivity which tests for the presence of the mutation in the lipopolysaccharide coating, and ampicillin resistance which tests for the presence of the R factor.[35] These spot tests were routinely used on bacteria stocks

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used in this research, and the results indicated no significant contamination of wild type bacteria or any other detectable change in the specifications of the strains. The bacteria experimental mutagenicity test methods used were standard procedures involving both positive and negative controls and other standard testing recommendations. It should be noted that while the spot and procedural tests are designed to detect most problems, they did not indicate defective tester bacteria in this study. However, the tests are not foolproof and changes in the tester bacteria remain a possibility for the problems observed.

Hypothesis 3

Hypothesis 3a—

This hypothesis is unlikely because the negative controls gave negative mutagenicity tests. Therefore, it is unlikely that bacterial contamination was occurring during the mutagenicity test.

Hypothesis 3b—

Hypothesis 3b was tested in the following ways: Glass vials and septum caps were autoclaved. A 2-mL sample was taken from the top of the HPLC resin column, and a 2-mL sample was taken from the bottom of the resin column. The HPLC column had been stripped with 6-M ammonium acetate eluent and reequilibrated with 0.015 M ammonium acetate eluent (pH 4.4) several days prior to sampling for the mutagenicity tests. The sample from the top of the column was slightly poisitive in both bacterial strains (TA-1535 and TA-1538) and the sample from the column bottom was positive in TA-1535 and negative but toxic in TA-1538 (Table 40, Series 1).

A blank chromatographic run was made with the standard linear HPLC gradient from 0.015 to 6.0 M ammonium acetate buffer using the same resin column used for HPLC of the concentrates of the effluent field samples. The 250 eluate fractions were combined into five larger pooled fractions: 1-50, 51-100, 101-150, 151-200, and 201-250. The eluate fractions were not lyophilized. The first fraction was negative in both strains. The remaining fractions showed increasing mutagenicity and toxicity (see Table 40, Series 2).

In each of the above cases, all samples were plated on test plates both with and without bacteria, and no bacterial contamination was detected. Therefore, no bacteria eluted from the column. Thus, hypothesis 3b was ruled out. The actual mutagenicity of the unlyophlized chromatographic fractions will be discussed below.

Hypothesis 3c—

The HPLC fractions from the above chromatographic blank test were lyhophilized and treated as in normal HPLC fraction preparation for mutagenicity testing and chemical characterization. All mutagenicity tests for the fractions were negative (Table 40, Series 3, old column). The resin from the column was reconditioned and repacked,

TABLE 40. MUTAGENICITY TESTS TO DETERMINE THE CAUSE OF ANOMALOUS NUMBER OF POSITIVE TESTS

Sample[a]	TA-1535	TA-1538
Series 1 (column samples - not lyophilized)		
Column top	Slightly positive	Slightly positive
Column bottom	Positive	Positive
Series 2 (chromatographic blank - not lyophilized)		
F-1 (1-50)	Negative	Negative
F-2 (51-100)	Positive	Positive and toxic
F-3 (101-150)	Positive	Positive and toxic
F-4 (151-200)	Positive and toxic	Positive and toxic
F-5 (201-250)	Positive and toxic	Positive and toxic
Series 3 (chromatographic blank - lyophilized)		
	Old column[b]	New column[c]
F-1 (1-50)	Negative	Negative
F-2 (51-100)	Negative	Negative
F-3 (101-150)	Negative	Negative
F-4 (151-200)	Negative	Negative
F-5 (201-250)	Negative	Negative
Series 4 (ammonium acetate solutions - not lyophilized)		
0.15 M	Negative	Negative
1.0 M	Positive	Positive
2.0 M	Positive and toxic	Positive and toxic
3.0 M	Positive and toxic	Positive and toxic
4.0 M	Positive and toxic	Positive and toxic
5.0 M	Positive and toxic	Positive and toxic
6.0 M	Positive and toxic	Positive and toxic

[a] In each case, all samples were plated on test plates both with and without bacteria, and no bacterial contamination was detected.

[b] Blank from Series 2 (above), i.e., resin colum used for HPLC separations of effluent field samples.

[c] Blank from reconditioned resin and freshly packed column.

and a blank chromatographic run made as above. All mutagenicity tests for the fractions were negative (Table 40, Series 3, new column). Therefore, hypothesis 3c was ruled out.

Hypothesis 4

Hypothesis 4a--

The negative results described in the hypothesis 3c section above tend to rule out buildup on the HPLC column resin and the subsequent elution of mutagenic constituents as a factor in the false positive problem. This hypothetical cause would be likely to cause sporadic false positive tests, not a series of false positives.

Hypothesis 4b--

The negative test results described in the hypothesis 3c section above tend to rule out the introduction of a chemical during fraction preparation as a viable cause for the false positives.

Because the fractions discussed in the hypothesis 3b section tested positive before lyophlization and therefore contained ammonium acetate, a series of ammonium acetate buffer solutions was prepared using standard chromatographic solutions from which the usual eluent buffers were prepared. The solutions prepared were 0.15, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 M ammonium acetate, pH 4.4. The 0.15 M solution was not mutagenic, but the remaining fractions were increasingly mutagenic and toxic (see Table 40, Series 4). Therefore, the mutagenicity of the solutions was attributable to the ammonium acetate or an impurity in the ammonium acetate. Consequently, in the lyophilization step to remove ammonium acetate buffer from the organic residues separated by HPLC, it is important to continue the lyophilization for a sufficient period to volatilize all the ammonium acetate. In general, the procedure was to lyophilize 24 hr longer than the last observation of ice or frozen solution in the material being lyophilized. This procedure was used in this research program for all preparations of HPLC pooled fractions for chemical characterization and mutagenicity testing.

It was concluded that the resin columns were not releasing mutagenic materials that created the false positive mutagenic tests.

Probable Cause

Although many spot checks (uv irradiation, crystal violet, and ampicillin tests for the possible reversion of tester strains to wild type were performed before and after the development of apparently false positive mutagenicity tests and the spot checks indicated no apparent problem with the bacterial tester strains, there continued to be indications of false positive results. When new stocks of tester strains were used in combination with filtration of the test samples through Swinex filters to ensure no bacterial contamination of the samples the mutagenicity test results appeared to be normal. The subsequent mutagenic activity testing in this research program was conducted using the new bacterial stock and filtration of the test sample. Consequently, no definitive conclusion was reached as to the cause of the false positive tests. However, there is a high probability that the false positive tests were caused by faulty test technique or contaminated bacterial tester stock.

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SECTION 9

SUMMARY

The effluents from nine wastewater treatment plants have been examined relative to (1) the chemical effects of the disinfectants chlorine, ozone, and ultraviolet light on nonvolatile organic constituents and (2) the formation of mutagenic constituents during disinfection. The wastewater treatment plants were selected for effluent sampling based on disinfection method, nature of the waste treated (i.e., domestic, commercial, light industry, heavy industry), type and quality of wastewater treatment, and geographical location relative to the research laboratory.

Evaluation of the chemical and toxicological effects of by-products of chlorination, ozonation, or uv irradiation in such complex matrices as effluents from wastewater treatment plants is a very arduous and complex problem. High-pressure liquid chromatography (HPLC) was the principal method used for examination of chemical effects on nonvolatile organics in concentrates of the effluents followed by gas chromatography/mass spectrometry (GC/MS) characterization of the separated constituents. Bacterial mutagenicity testing was used as the principal endpoint for determining biological effects.

Initially, effluents from two local wastewater treatment plants were used to evaluate experimental methodology and to determine disinfection effects under controlled laboratory conditions. These treatment plants routinely disinfected effluents by chlorination. However, comparative studies of chemical effects were made by sampling the effluents prior to chlorination and then disinfecting the effluents in the laboratory with ozone or uv-light irradiation.

Seven additional wastewater treatment plants were selected for effluent characterization. The majority of these plants used an advanced wastewater treatment incorporating ozonation or uv irradiation and were operated under the auspices of an EPA program for the evaluation of wastewater treatment schemes. Effluents from the plants permitted the comparison of general chemical effects of disinfection methods and initial assessment of their potential for production of mutagenic substances.

METHODS DEVELOPMENT

Investigation of disinfection effects on wastewater effluent constituents of low volatility was accomplished using the following general methodology: (1) grab samples of 40 L; (2) air transportation to ORNL of samples at -40 to 0°C or ground transportation at ambient temperatures; (3) concentration of the undisinfected (control) effluent and disinfected effluents by 1000- to 3000-fold using vacuum concentration (generally freeze drying); (4) assaying the concentrate for mutagenic activity using bacterial mutagenicity tests; (5) separation of the nonvolatile organic constituents in the concentrate by high-pressure anion exchange chromatography; (6) assaying the separated chromatographic constituents (in collective subsamples of HPLC eluate fractions) for mutagenic activity using bacterial mutagenicity tests; (7) chemical characterization of the separated chromatographic constituents using uv spectrometry, gas chromatography, and gas chromatography/mass spectrometry.

Vacuum distillation was the concentration method of choice in previous studies involving relatively small volumes of effluents. However, vacuum distillation using available apparatus was very labor intensive; consequently, several different concentration methods were evaluated. Lyophilization (freeze drying) was selected as the optimum concentration method because it appeared to produce concentrates equivalent to vacuum distillation as determined by HPLC and because it required much less labor and attention regarding machine operation. Adsorption on XAD resins was investigated briefly and appeared to be a viable technique for concentration of uv-absorbing and mutagenic constituents. Resin adsorption should be evaluated more thoroughly.

Considerable reduction in HPLC chromatographic time was accomplished by pretreating and packing anion exchange resin columns with ethanol. However, in this study it was observed that phosphate-containing organic constituents may be degraded by passage through the anion exchange column. This matter needs thorough investigation.

Fluorescence measurement is potentially a very sensitive chromatographic detection method. It was evaluated in this study and significantly increased the number of apparent constituents separated by HPLC. A large number of nonvolatile organic constituents in wastewater effluents are fluorescent (λ_{ex}, 360 nm; λ_{em}, >400 nm). Consequently, a fluorometer is now being used routinely at ORNL as a detector for HPLC separations.

CHEMICAL EFFECTS

By using ozone dosages sufficient to achieve customary disinfection levels in the initial laboratory studies, it was determined that ozonation destroys and also produces many HPLC chromatographic constituents presumably by oxidation reactions. With increasing ozone dosage, the number and quantity of ozone-produced constituents reaches a maximum then decreases. It is apparent that ozonation would ultimately destroy most of the chromatographic constituents, although the required ozone dosage would be much greater than that required for normal disinfection. It is hypothesized that some of the increase in chromatographic constituents detected in the HPLC chromatograms of effluents ozonated at intermediate dosages is due to oxidation and degradation of larger nonvolatile organics which either do not chromatograph on the HPLC or elute with the first chromatographic peak. A good example of this effect is the occurrence of histidine in ozonated but not in undisinfected effluents of two separate collections at Estes Park. It appears that histidine or a histidine surrogate was released by ozonation from peptides or proteins present in the wastewater before treatment.

Similarly, it was observed that disinfection with chlorine destroys constituents represented by HPLC chromatographic peaks in undisinfected (control) effluents and produces others. Previous work had indicated that chloroorganics are produced although the principal organic reaction of aqueous chlorine in wastewater effluents was oxidation of the organics.[4] As in the case with ozone, it was shown that, with sufficiently high chlorine dosages, most of the HPLC chromatographic constituents could be destroyed.

In laboratory experiments, it was determined that irradiation with ultraviolet light at approximately 25,000 to 30,000 $\mu W \cdot s/cm^2$ achieved disinfection levels in effluents comparable to that usually achieved with 0.5 to 1 mg/L chlorine or ozone dosages of 6 mg ozone per liter effluent. At this uv-irradiation level, relatively few chemical effects were observed in the laboratory with either primary or secondary effluents. At irradiation levels of 60,000 $\mu W \cdot s/cm^2$, some destruction of a few peaks was seen and a few small chromatographic peaks were apparently produced. However, uv irradiation at the Northwest Bergen County Wastewater Treatment Plant was observed to have some chemical effects possibly because of higher irradiation dosages. For example, one relatively large chromatographic peak was produced by uv irradiation. The identity of this peak was not established although it contained several constituents such as *o*-hydroxyphenylacetic acid, oxalic acid, phosphate, and an aliphatic hydrocarbon. In addition, it was determined that uv irradiation did destroy some mutagenic substances that were present in the undisinfected (control) effluent. Therefore, although relatively slight in comparison with ozone and chlorine, some chemical effects on nonvolatile organics in wastewater effluents may be observed when the effluent is disinfected with uv irradiation.

FIELD SAMPLE STUDIES

Lyons Bend Fourth Creek Wastewater Treatment Plant

Concentrates of both the undisinfected and chlorinated effluents from the Lyons Bend wastewater treatment plant were weakly mutagenic. This treatment plant used a conventional activated-sludge secondary treatment to treat waste that was principally domestic with about 20% commercial and light industry. Mutagenicity tests of the nonvolatile organic constituents separated by HPLC from the undisinfected effluent concentrate indicated that two fractions were weakly mutagenic. There were one positive and eight weakly positive fractions for the concentrate of the chlorinated effluent (residual chlorine, 0.9 mg/L). It was concluded that chlorination of the Lyons Bend secondary effluent destroyed some mutagenic constituents but also produced somewhat more total mutagenic activity and numbers of mutagenic constituents. No compounds known to be mutagens were identified in the mutagenic HPLC eluate fractions.

Moccasin Bend Wastewater Treatment Plant

The concentrate of the undisinfected effluent from the Moccasin Bend wastewater treatment plant was strongly mutagenic in the first sample collection (1978) and weakly mutagenic in the second collection (1979). Chlorination essentially eliminated the mutagenic activity for the fall 1978 sample (residual chlorine, <0.2 mg/L) but increased it very significantly for the summer 1979 sample (residual chlorine, <0.2 mg/L). The Moccasin Bend treatment plant used conventional activated-sludge secondary treatment to process waste from a large industrial complex (the waste is 65% industrial and 35% residential). Mutagenicity tests of HPLC fractions for concentrates of each unchlorinated and chlorinated effluent revealed several mutagenic fractions. One positive and eight slightly positive fractions were found for the 1978 unchlorinated effluent and nine slightly positive for the corresponding chlorinated effluent. However, the nature of the mutagenic constituents changed from principally mutagenic in TA-1535 (a base pair substitution mutagen detector) for the undisinfected effluent to mutagenic in TA-1538 (a frame-shift mutagen detector) for the chlorinated effluent. One positive and eight slightly positive fractions were found for the unchlorinated 1979 effluent concentrate and one positive and two slightly positive fractions for the corresponding chlorinated effluent concentrate. Two of the slightly mutagenic fractions were common in both the chlorinated and unchlorinated concentrates. It was concluded that chlorination of the Moccasin Bend secondary effluent destroyed some mutagenic constituents and produced others. For each case, the total mutagenic activity detected in the HPLC eluate fractions was considerably less than that determined for the corresponding concentrates before chromatography. Therefore, apparently many mutagenic constituents in the effluents are either not chromatographed (i.e., absorbed irreversibly) or may be decomposed on the anion exchange resin during chromatography. No compounds known to be mutagens were identified in the mutagenic HPLC eluate fractions.

Marlborough Easterly Advanced Waste Treatment Plant

The concentrate of the undisinfected, nitrified effluent (3/12/79) effluent was mutagenic, but the concentrates of both the ozonated, and chlorinated, nitrified effluents were not mutagenic. However, mutagenic fractions were isolated from all three concentrates. The Marlborough wastewater treatment plant used a two-stage activated-sludge treatment, and chlorination for disinfection. The first stage of activated sludge treatment removed BOD and phosphorous and the second stage was optional nitrification. For comparative studies an ozonation pilot plant was used to disinfect a portion of the effluent. The waste treated was principally domestic. One slightly positive mutagenic HPLC fraction was separated from the concentrate of the undisinfected effluent. Four positive and one slightly positive mutagenic HPLC fractions were separated from the concentrate of the ozonated effluent. Two positive mutagenic HPLC fractions were separated from the chlorinated effluent concentrate. For the second set of effluent samples, nine slightly mutagenic HPLC fractions were separated from the concentrate of the undisinfected effluent. For the ozonated effluent concentrate two slightly positive mutagenic HPLC fractions were detected. Four positive and three slightly mutagenic HPLC fractions were separated from the chlorinated effluent concentrate.

For both sets of samples, nonvolatile mutagenic constituents were separated from the concentrates of the undisinfected effluents. Ozonation apparently produced a significant increase in the mutagenicity in the first set but considerably less in the second set with accompanying destruction of mutagenic constituents detected in the undisinfected effluent. Chlorination produced much more mutagenic activity in the effluent collected during the second period than during the first period. For the chlorinated effluent in the second collection period (9/19/79), three of the mutagenic HPLC fractions were common with the undisinfected effluent. Thus, there appears to be considerable variability between samples collected from the same wastewater treatment plant. No compounds known to be mutagens were identified in the mutagenic HPLC fractions.

Upper Thompson Sanitation District

The Estes Park wastewater treatment plant used activated sludge, nitrification, filtration, and ozonation to treat principally domestic waste. None of the concentrates of undisinfected or ozonated effluents from three field collections were mutagenic. However, two slightly mutagenic HPLC fractions were isolated from the undisinfected nitrified effluent of the third set of samples, and one positive and four slightly positive mutagenic fractions were separated from the corresponding ozonated, nitrified effluent. None of the mutagenic fractions were common to both effluents. Thus, ozonation clearly destroys some mutagenic constituents and also produces others. This also is another example of mutagenic fractions separated from effluent concentrates that had tested negative. No compounds known to be mutagens were identified in the mutagenic fractions.

Taft Center Pilot Wastewater Treatment Plant

The Taft Center pilot plant used conventional activated-sludge secondary treatment to treat waste that was principally domestic with some industrial input. None of the concentrates of undisfinected or ozonated effluents for both field samples collected at the Taft Center pilot plant tested mutagenic. Mutagenicity tests of the HPLC eluate fractions revealed two slightly positive HPLC fractions in the undisinfected effluent and, also, two different slightly positive HPLC fractions in the ozonated effluent. Thus, mutagenic fractions were again recovered from apparently nonmutagenic concentrates. It is concluded from these results that ozonation destroyed mutagenic constituents and produced others. No compounds known to be mutagens were identified in the mutagenic fractions.

Meander Creek Wastewater Treatment Plant

The Meander Creek Wastewater Treatment Plant used two-stage oxygenation to remove BOD and for nitrification, tertiary treatment to remove phosphorous, filtration, and ozonation. Mutagenicity tests were negative for the concentrates of the unozonated and ozonated effluents from both sets of samples. Two HPLC fractions from the concentrate of the unozonated effluent of the first set tested slightly mutagenic, and seven fractions from the unozonated effluent of the second set tested mutagenic (one positive and six slightly positive). The mutagenic test data for the ozonated effluent samples of the second set were not acceptable because of reversion of the bacterial test strains to wild type. No compounds known to be mutagens were identified in the mutagenic HPLC fractions.

Northwest Bergen County Wastewater Treatment Plant

The Northwest Bergen County Wastewater Treatment Plant used conventional secondary treatment of activated sludge and chlorination to treat waste that was principally domestic. As a pilot plant study, approximately half the effluent was disinfected by uv irradiation and the remainder was disinfected by chlorination. Concentrates of the undisinfected and uv-irradiated effluent samples of the first set tested negative for mutagenicity. In the second set, the concentrate of the undisinfected effluent tested negative, but the concentrate of both the uv-irradiated and the chlorinated effluents tested slightly mutagenic. Four HPLC fractions separated from the concentrate of the undisinfected effluent were found to be mutagenic (three positive and one slightly positive). No fractions separated from the concentrates of either the uv-irradiated or the chlorinated effluents were mutagenic. Thus, both uv irradiation and chlorination apparently produced some nonvolatile mlutagenic components that were deactivated by anion exchange chromatography and, also, destroyed the chromatographic mutagenic constituents in undisinfected effluent. No compounds known to be mutagens were identified in the mutagenic HPLC fractions.

IDENTIFIED CONSTITUENTS

A total of 103 constituents were identified in the concentrates of the control and disinfected effluents. Seventy-seven constituents were identified in mutagenic HPLC fractions; 48 were identified in nonmutagenic fractions; 22 compounds were common in both mutagenic and nonmutagenic fractions. The identified constituents are summarized according to chemical class in Table 41.

MUTAGENIC NONVOLATILE ORGANIC CONSTITUENTS

Mutagenicity of some constituents in concentrates of several wastewater effluents has been demonstrated and, in spite of imperfections in the testing technology, the reality of the effect is not in doubt. It is clear that mutagenic components can occur in wastewater effluents before disinfection. It is also clear that disinfection by either chlorine or ozone can lead to an increase in the number of mutagenic materials present in these effluents. However, the story is far from simple. Sometimes the mutagenicity of a sample is increased by disinfection, and sometimes the reverse is true. The data obtained in the HPLC separations suggest the possibility of complex interactions between various components of the effluent concentrates. We do not understand all of the factors that lead to the effects observed in these samples. In several experiments the data suggested that some of the mutagenic agents are either lost or inactivated in passage through the strongly basic anion exchange HPLC column. In several other experiments, the data indicated that separation of constituents from the original mixture of nonvolatile constituents in the effluent concentrate permitted their mutagenicity to be detected, i.e., the mutagenic activity of the substance had been masked by the toxicity of the total mixture.

To determine whether the mutagenic activity in chlorinated effluents was associated with constituents containing active chlorine, a very mutagenic chlorinated effluent was treated with thiosulfate prior to concentration. The mutagenic activity was not inactivated by thiosulfate. Therefore, the mutagenicity of the effluent was not attributable to reactive chlorine, e.g., organic chloramines (N-chloroorganic compounds).

TABLE 41. SUMMARY OF IDENTIFIED COMPOUNDS IN HPLC FRACTIONS OF EFFLUENT CONCENTRATES

Alcohols

- 4-Butoxy-1-butanol[a]
- Cyclobutanol[a]
- 2,4-Dimethyl-3-pentanol[a]
- 4-Methyl-2-propyl-1-pentanol[a]

Aliphiatic Acids

- Diglycolic acid[a,b]
- 2,2-Dimethyl-3-hydroxybutyric acid[b]
- 3,2-Dimethyl-3-hydroxybutyric acid[a]
- Glutamic acid[a]
- 3-Hydroxybutyric acid[b]
- 2-Hydroxyisobutyric acid[a,b]
- 3-Hydroxyisovaleric acid[b]
- Isovaleric acid[a]
- Ketoglutaric acid[a]
- Lactic acid[a,b]
- Malonic acid[b]
- 2-Methyl-2-hydroxybutyric acid[a]
- Muconic acid[a]
- Oxalic acid[a,b]
- Propanoic acid[a]

Amines

- Canavanine[b]
- *N,N'*-diethyl-*N,N'*-dimethyl-*p*-phenylenediamine[a]

Amino Acids

- Alanine[a,b]
- Aminoisobutyric acid[b]
- Aspartic acid[b]
- Cystine[a]
- Glycine[a]
- Histidine[a]
- Homocystine[a]
- Phenylalanine[a]
- Serine[a]
- Tyrosine[a]

Aromatic Acids

- Benzoic acid[a,b]
- 4-Chlorobenzoic acid[a,b]
- Dichlorobenzoic acid[a]
- O-hydroxyphenylacetic acid[b]
- Phthalic acid[a]
- Phenylpuruvic acid[a]
- 2-Pyridinecarboxylic acid[a]

Carbohydrates

- Erythrose[a,b]
- Erythrono-1,4-lactone[b]
- Galactose[b]
- Glucose[b]
- Gluconic acid[b]
- 1-Methylmannofuranoside[a]
- 2-Methylxylopyranoside[a]
- Rhamnose[b]
- Ribino-1,4-lactone[b]
- Threono-1,4-lactone[a,b]
- Xylono-1,4-lactone[a]
- Xylose[a,b]

Epoxide

- 2-Methyl-3-propyl oxirane[a]

Fatty Acids

- Linoleic acid[a]
- Myristic acid[b]
- Oleic acid[b]
- Palmitic acid[a,b]
- Stearic acid[a,b]
- Tetradecanoic acid[b]

TABLE 41. (continued)

Hydrocarbons

- Decane[b]
- 2,4-Dimethylhexane[a]
- Dodecane[a]
- Ethylindan[a]
- 4-Ethyltetradecane[b]
- Methyleicosane[a]
- 5-Methyl-5-ethyldecane[b]
- 2-Methylhexadecane[b]
- 2-Methyloctane[a]
- 2-Methyl-1,3,6-trioxocane[a]
- Nordodecane[a]
- Pentacosane[a]
- Pentadecane[b]
- 2,2,3,3-Tetramethylhexane[a,b]

Phenols

- Phenol[a,b]

Phthalates

- Di-*n*-Butylphthalate[b]
- Diethylphthalate[b]
- Diisobutylphthalate[a,b]

Polyols

- Cyclohexane-1,4-diol[a]
- Dihydroxyacetone[b]
- 2,5-Dimethyl-3,4-hexanediol[a]
- Erythritol[a,b]
- Ethylene glycol[a,b]
- Glucitol[a,b]
- Glycerol[a,b]
- Inositol[a]
- 1,3-Propanediol[a,b]
- Propylene glycol[a]
- Xylitol[a]
- Threitol[a,b]

Purines and Pyrimidines

- Adenosine[a]
- Caffeine[a]
- 6-Dimethyladenosine[a]
- 5-Hydroxyuridine[a]
- Thymine[a]
- Uracil[a,b]

Miscellaneous

- 2-Aminoethanol[a]
- *O*-Decylhydroxyl amine[a]
- 2,4-Dimethyl-2,4-disilapentane[a]
- 3,7-Dioxa-2,8-disilanonane-2-one-2,2,8,8-tetramethyl[a]
- L-α-Glycerophosphate[a]
- Phenylacetamide[a]
- Phenylpyruvic oxime[a]

- 1,2,3-Trimethoxypentane[b]
- 3,6,9-Trioxa-2-silaundecane-2-3-dimethyl[a]
- Urea[a]

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[a] Identified in mutagenic HPLC fraction.
[b] Identified in a nonmutagenic HPLC fraction.

Most of the mutagens detected in the concentrates of the wastewater effluents were direct acting; that is, there was a decrease in mutagenicity by addition of S-9 (metabolic activation enzymes). Both base pair substitution and frame-shift mutagens occurred in the wastewaters examined, but the base pair mutagens were more frequent. For many of the materials present in the effluents, strain TA-1535 was more sensitive than strain TA-100 in detecting the base pair substitution mutagens.

The mutagenic tests and chemical characterization studies were conducted with 1000 to 3000-fold concentrates of the wastewater effluents. Mutagenic activity was not demonstrated with the original effluent samples.

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APPENDIX

CHEMICAL, PHYSICAL, DISINFECTION DATA FOR WASTEWATER TREATMENT PLANTS AND EFFLUENT SAMPLES

Chemical, physical, and disinfection data for the effluent samples used in this research program and general treatment plant data are given here. The data were provided by the operators of the respective wastewater treatment plants or associated contractors assisting the EPA in evaluating the plants. Chemical and treatment data are generally limited to the analyses routinely performed during the course of plant operation.

OAK RIDGE WASTEWATER TREATMENT PLANTS

The Oak Ridge West Wastewater Treatment Plant uses mechanical grit removal, primary treatment with anaerobic sludge digestion to process waste from the west side of Oak Ridge, Tennessee. It serves approximately 18,000 people, including some business and commercial enterprises, and is designed to process waste at 0.22 m^3/s (5.0×10^6 gal/day). The effluent from the primary settling basin is routinely chlorinated before discharge to the East Fork of Poplar Creek, which flows into the Clinch River. Effluent data provided by the treatment plant operator are given in Table 42.

The Oak Ridge East Wastewater Treatment Plant uses mechanical grit removal, primary settling, secondary treatment by activated sludge, settling, and anaerobic-sludge digestion to process waste from the east side of Oak Ridge, Tennessee. It serves approximately 7000 people, including some light industry (e.g., a machine shop), and is designed to process waste at 0.066 m^3/s (1.5×10^6 gal/day). The effluent from the secondary settling basin is routinely chlorinated before discharge to the Clinch River. Effluent data provided by the treatment plant operator are given in Table 43.

LYONS BEND FOURTH CREEK WASTEWATER TREATMENT PLANT

The Lyons Bend Fourth Creek Wastewater Treatment Plant uses screening for grit removal, primary settling, and secondary treatment by activated sludge followed by settling, and anaerobic-sludge digestion to process waste from the west side of Knoxville, Tennessee. It serves approximately 82,000 people, and the waste

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TABLE 42. EFFLUENT DATA FOR PRIMARY EFFLUENT SAMPLES COLLECTED AT OAK RIDGE WEST WASTEWATER TREATMENT PLANT

Date	Flow[a] [m^3/s(MGD)]	BOD[b] (mg/L)	pH	Temperature (°C)	Residual chlorine[c] (mg/L)
9/27/77	0.15 (3.5)	13	7.0	20	1.1
12/12/77	0.051 (1.4)	2.5	7.2	15	0.9
5/19/78	0.16 (3.6)	6.0	7.2	17	0.8
7/17/78	0.12 (2.7)	80	7.5		1.3
11/29/78	0.10 (2.4)	5.0	7.0	17	0.9
11/30/78	0.10 (2.4)		7.0	18	

[a] Average flow.
[b] For effluent prior to chlorination.
[c] Residual data given for chlorinated effluent. Undisinfected effluents sampled for ozonation and uv-irradiation studies were collected before chlorination.

TABLE 43. EFFLUENT DATA FOR SAMPLES COLLECTED AT OAK RIDGE EAST WASTEWATER TREATMENT PLANT

Date	Effluent type	Flow [m^3/s(MGD)]	BOD$_5$[a] (mg/L)	pH	Temperature (°C)	Residual chlorine[b] (mg/L)
2/7/77[c]	Secondary	0.053 (1.2)	29	6.8	9	0.8
4/11/77[c]	Secondary	0.074 (1.1)	5.0	6.9	18	1.1
5/18/77[c]	Secondary	0.057 (1.3)	6.5	6.8	22	0.4
6/19/77	Primary	0.051 (1.4)	34	6.9		
8/2/77	Secondary	0.070 (1.6)	42	6.8	23	0.6
8/9/77	Secondary	0.074 (1.7)	39	7.1	26	0.4
9/27/77	Secondary	0.083 (1.9)	8.0	6.9	22	0.5
10/5/77[c]	Secondary	0.070 (1.6)	30	6.8	22	0.8
1/31/78	Secondary	0.057 (1.3)	8.0	7.5		0.8
2/8/78[c]	Secondary	0.048 (1.1)	13	6.8	10	0.6
2/21/78	Secondary	0.044 (1.0)	24	6.7	11	0.6
7/13/78	Secondary	0.031 (0.7)	19	6.8	24	0.7

[a] Prior to chlorination.

[b] For chlorinated effluent. Undisinfected effluents sampled for ozonation and uv-irradiation studies were collected before chlorination.

[c] Data not taken on day of collection. Values represent the average of values for day preceeding and day following sample collection.

treated is approximately 80% domestic and 20% business and light industry. The plant processes waste at flows up to 0.44 m^3/s (10 x 10^6 gal/day). The effluent from the secondary settling basin is routinely chlorinated before discharge to the Fort Loudon Lake (Tennessee River). Two collections of the control (secondary effluent before chlorination) and chlorinated secondary effluents were made on 5/3/78 and 10/25/78. Effluent data provided by the treatment plant operator are given in Table 44.

MOCCASIN BEND WASTEWATER TREATMENT PLANT

For comparative purposes, it was of interest to examine the undisinfected and chlorinated secondary effluents from a relatively large [2.2 to 4.4 m^3/s (50 to 100 Mgd)] wastewater treatment plant treating industrial wastes in addition to residential wastes. A telephone survey was conducted to determine the locations of such plants within a convenient geographic radius of ORNL. Generally, a relatively large metropolitan area (popoulation 500,000) is required for wastewater volumes to be in the 2.2 to 4.4 m^3/s (50 to 100 Mgd) range. The treatment plants surveyed were in Atlanta, Georgia; Birmingham, Alabama; Charlotte, North Carolina; Cincinnati, Ohio; Indianapolis, Indiana; Jacksonville, Florida; Little Rock, Arkansas; Louisville, Kentucky; St. Louis, Missouri; and Chattanooga, Memphis, Knoxville, and Nashville, Tennessee. Of these, the Moccasin Bend Wastewater Treatment Plant located at Chattanooga was selected for effluent sampling.

The Moccasin Bend Wastewater Treatment Plant uses bar screening, grit removal, primary settling, and secondary treatment by activated sludge followed by settling to process waste from the industrial section of Chattanooga, Tennessee. It serves approximately 155,000 people, and the waste treated is about 35% domestic and 65% industrial. The plant is designed to process waste at flows up to 3.5 m^3/s (80 x 10^6 gal/day). The effluent from the secondary settling basin is routinely chlorinated before discharge to the Tennessee River several miles downstream from Chattanooga. Two collections of the control (secondary effluent before chlorination) and chlorinated secondary effluents were made on 9/27/78 and 7/17/79. Effluent data provided by the treatment plant operator are given in Table 45.

MARLBOROUGH EASTERLY ADVANCED WASTE TREATMENT PLANT

The Marlborough Easterly Advanced Waste Treatment Plant serves approximately 20,000 people in the Marlborough, Massachusetts area. The treated waste is principally residential (99%) with only slight commercial input. The treatment consists of grit removal, primary

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TABLE 44. CHEMICAL AND DISINFECTION DATA FOR EFFLUENT SAMPLES COLLECTED AT LYONS BEND FOURTH CREEK WASTEWATER TREATMENT PLANT

	Sample Set 1	Sample Set 2
Date	5/3/78	10/25/78
pH	7.3[a]	7.0[a]
Flow, m^3/s (MGD)	0.43 (10)[a]	0.33 (7.5)[a]
COD, mg/L	140[a]	
NH_3-N, mg/L	17[a]	11[a]
Suspended solids, mg/L	60[a]	35[a]
Total solids, mg/L	500[a]	550[a]
Dissolved oxygen (DO), mg/L	1.2[a]	1.5[a]
BOD_5, mg/L	34[a]	
Residual chlorine, mg/L	0.9[a]	1.5[a]
Fecal coliform, no./100 mL	135[a]	115[a]

[a] Chlorinated effluent.

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TABLE 45. ANALYTICAL DATA FOR EFFLUENT SAMPLES COLLECTED AT MOCCASIN BEND WASTEWATER TREATMENT PLANT

	Sample Set 1	Sample Set 2
Date	9/27/78	7/17/79
Flow, m^3/s (MGD)	1.62 (37)[a]	1.71 (39.3)[a]
BOD_5, mg/L	84[a]	97[a]
COD, mg/L	228[a]	342[a]
pH	8.1[a]	7.8[a]
NH_3-N, mg/L	3.8[a]	6.6[a]
TKN, mg/L	14[a]	
Suspended solids, mg/L		194[a]
Settleable solids, mg/L		15[a]
Volatile solids, mg/L		118[a]
Residual chlorine, mg/L	< 0.2[a]	< 0.2[a]

[a] Chlorinated effluent.

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settling, two-stage activated-sludge treatment with chemical addition in the first stage to remove phosphorous, secondary settling, optional nitrification to remove ammonia, followed by chlorination or ozonation of the effluent before discharge to Hop Brook, which flows through a series of impoundments to the Sudbury River. The treatment plant is designed to process waste at flows of 0.13 m^3/s (3×10^6 gal/day). Two collections of the control (nitrified effluent before disinfection), chlorinated nitrified, and ozonated nitrified effluents were made on 3/12/79 and 9/19/79. Effluent data provided by the treatment plant operator are given in Table 46.

UPPER THOMPSON SANITATION DISTRICT

The Upper Thompson Sanitation District, Estes Park, Colorado, serves approximately 12,000 people during tourist seasons and about 2,000 people during periods with little tourist activity. The treatment scheme consists of mechanical grit removal, activated-sludge treatment, secondary clarification, nitrification (ammonia removal), multimedia filtration (gravel, sand, and anthracite coal), and ozonation of the effluent before discharge to the Big Thompson River. The treatment plant processes waste at flows of approximately 0.05 m^3/s (1×10^6 gal/day). The waste treated is essentially all domestic. Three collections of the control (trimedia filter effluent before ozonation) and ozonated effluents were made on 10/19/77, 7/8/78, and 8/6/79. Effluent data provided by the treatment plant operator are given in Table 47.

TAFT CENTER PILOT WASTEWATER TREATMENT PLANT

The Taft Center Pilot Wastewater Treatment Plant, operated by the U.S. Environmental Protection Agency, Cincinnati, Ohio, was designed to study advanced wastewater treatments. The pilot plant consisted principally of a primary clarifier, conventional activated-sludge secondary treatment, with optional filtration of the effluent. For this study, the effluent was disinfected by ozonation. The effluent from the pilot plant was discharged back to the municipal sewer system. The waste treated was principally residential with some industrial input. The pilot plant generally processed waste at a flow of 3.1×10^{-3} m^3/s (50 gal/min) and the effluent was disinfected at 1.3×10^{-3} m^3/s (20 gal/min). Two collections of the control (unozonated) and ozonated effluents were made on 9/12/77 and again on 5/24/78.

Sample 1

Conditions and results for ozonation of secondary effluent collected on 9/12/77 are as follows.

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TABLE 46. PHYSICAL, CHEMICAL, AND DISINFECTION DATA FOR EFFLUENT SAMPLES COLLECTED AT MARLBOROUGH EASTERLY ADVANCED WASTEWATER TREATMENT PLANT

	Sample Set 1					Sample Set 2	
	Raw	Primary	Secondary	Nitrified	Final	Nitrified	Final
Date	3/12/79	3/12/79	3/12/79	3/12/79	3/12/79	9/19/79	9/19/79
Flow, m^3/s (MGD)	0.21 (4.8)					0.092 (2.1)	
COD, mg/L	190	400	60		29		
BOD_5, mg/L	71	150	16		3.0[a]		1.5[a]
Total suspended solids (TSS), mg/L	130	250	20		6.8[a]		1.2[a]
Volatile suspended solids (VSS), mg/L	91	190	18		6.4[a]		1.0[a]
Total P, mg/L	4.0	8.3	0.9		0.4[a]		
NH_3-N, mg/L	9.1	13	9.3		0.08[a]		
Settleable solids, mL	4.5	5	0.1		0[a]		
pH	6.8	7.0	7.5		6.6[a]		7.5[a]
Total alkalinity, mg/L			91		13[a]		90[a]
Specific conductivity							640[a]
Chlorinated effluent							
Chlorine dose, mg/L					3.0		7.0
Residual chlorine, mg/L					2.0		3.0
Total coliform, no./100 mL			1.2×10^6	8,000	13	29,000	180
Fecal coliform, no./100 mL			92,000		<1	2,100	2.2
Ozonated effluent							
Ozone dose, mg/L					17.3		32.7
Adsorbed ozone, mg/L					12.2		17.7
Total coliform, no./100 mL			1.2×10^6	8,000	8	29,000	2.2
Fecal coliform, no./100 mL			92,000		<1	2,100	2.2

[a] Chlorinated nitrified effluent.

TABLE 47. CHEMICAL AND DISINFECTION DATA FOR THE UPPER THOMPSON SANITATION DISTRICT WASTEWATER TREATMENT PLANT DURING PERIODS OF EFFLUENT SAMPLE COLLECTION. OCCASIONALLY ANALYSES WERE NOT RUN ON THE SAMPLING DATE, AND THE VALUES SHOWN WERE AVERAGED FROM AVAILABLE DATA FOR THE WEEK DURING WHICH SAMPLES WERE COLLECTED.

	Sample Set 1		Sample Set 2				Sample Set 3				
	Trimedia filter effluent	Ozonator effluent	Plant influent	Activated-sludge effluent	Nitrification tower effluent	Ozonator effluent	Plant influent	Activated sludge effluent	Nitrification tower effluent	Trimedia filter effluent	Ozonator effluent
Date	10/19/77	10/19/77	7/8/78	7/8/78	7/8/78	7/8/78	8/6/79	8/6/79	8/6/79	8/6/79	8/6/79
Flow, M^3/s (MGD)		0.014 (0.32)				0.044 (1.0)					0.03 (0.70)
pH	6.3	6.2		6.7	6.9	6.9		6.5		6.7	
DO, mg/L	6.0	7.0		2.5						7.4	
BOD_5, mg/L	1.8	3.2	420	37	94	30	420	78	85	21	31
COD, mg/L	28	36									
TKN-N, mg/L	1.1	1.4		29	35						
NH_3-N, mg/L	0.01	0.01	30	18	9.1	19	27	21	22		13
NO_2-NO_3-N, mg/L	17	15	0.14	1.8	7.4						
Total P, mg/L	4.2	3.6				5.7	12				6.3
Alkalinity, mg/L	13	7.6		164	95						
TSS, mg/L	2.3	3.7	250		22	5.3	360	32	26	3.7	5.4
VSS, mg/L	1.0	1.3									
Ozone dose, mg/L		5.0				5.0					6.0
Total coliform (MF[a]), no./100 mL	9.2×10^3	42				5.2×10^5					
Total coliform (MPN[b]), no./100 mL	140	33									
Fecal coliform (MF), no./100 mL	540	1				1.5×10^5				2.5×10^5	2.2×10^5
Fecal coliform (MPN), no./100 mL	140	13									

[a] Membrane filter.
[b] Maximum probable number.

The secondary effluent from the Taft Center activated-sludge pilot plant was fed at a rate of 76 L/min to the diffuser columns. The diffuser columns consisted of three columns, each operating with the liquid flowing into the top and the ozone-enriched gas entering at the bottom through porous stone diffusers. Liquid residence time in each column was 3.3 min, giving a total residence time of approximately 10 min. Approximately 50% of the ozone-enriched gas was fed into the first column, with the remaining 50% equally divided between columns 2 and 3.

The ozone system operating conditions are shown in Table 48. The chemical characterization of the effluent entering and leaving each diffuser column is shown in Table 49.

The bacteriological results from the experiment are shown in Table 50. The membrane filter (MF) procedure was used in their determination.[30]

Sample 2

The conditions for ozonation of the second set of samples of CSE secondary effluent (5/24/78) are given in Table 51.

MEANDER CREEK WASTEWATER TREATMENT PLANT

The Meander Creek Wastewater Treatment Plant, Mineral Ridge, Ohio, serves approximately 43,000 people. The treated waste is about 95% domestic and 5% industrial in origin. The treatment consists of comminution, aerated grit removal, first-step oxygenation for BOD removal, settling, second-step oxygenation for nitrification to remove ammonia, settling, tertiary treatment to remove phosphorous, addition of CO_2 to adjust the pH, dual-media filtration (sand and anthracite coal), followed by ozonation of the effluent. The effluent is discharged to Meander Creek, which flows into the Mahoning River. The treatment plant processes waste at flows of 0.088 m^3/s (2×10^6 gal/day). Two collections of the control (nitrified effluent before ozonation) and ozonated nitrified effluents were made on 6/15/76 and 10/5/78. Effluent data provided by the treatment plant operator are given in Table 52.

NORTHWEST BERGEN COUNTY WASTEWATER TREATMENT PLANT

The Northwest Bergen County Wastewater Treatment Plant, Waldwick, New Jersey, serves approximately 40,000 people. The treated waste is essentially all domestic in origin with only slight commercial input. The wastewater treatment consists of preliminary screening, comminution, primary settling, and conventional secondary treatment by activated

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TABLE 48. OZONE OPERATING CONDITIONS
(CSE Sample Set 1)

Item	Ozonator	Diffusers 1	Diffusers 2	Diffusers 3
Power, W	750			
Pressure, kPa	62			
Gas dew point, °C	-77			
Gas temperature, °C	29			
Gas flow, L/min		40	21	21
O_3 conc. in, mg/L		8.3	8.3	8.3
O_3 conc. out, mg/L		0.3	1.2	2.6
O_3 utilized, %		96	86	69
Secondary effluent (SE), L/min		76	76[a]	76[a]
O_3 residual, mg O_3/L SE[b]		0.2	0.2	0.2
O_3 applied dosage, mg O_3/L SE		4.2	2.1	2.1
O_3 consumed, mg O_3/L SE		3.8	1.8	1.4
SE temp, °C		23	23[a]	23[a]
Gas/liquid, L air/L SE		0.53	0.28	0.28

[a] System operated in series.

[b] Ozone residual analyses is suspect since past history indicates a rise in residual as effluent flows from column 1 through column 3.

TABLE 49. SECONDARY EFFLUENT CHEMICAL CHARACTERISTICS (CSE Sample Set 1)

Item	Influent	Diffusers 1	Diffusers 2	Diffusers 3
Total COD, mg/L	52	50	59	59
Soluble COD, mg/L	50	52	49	48
TSS, mg/L	7.0	6.2	5.8	3.4
VSS, mg/L	6.2	4.8	3.6	2.2
TOC, mg/L	28	27	27	25
TKN-N, mg/L	28.2	29.5	29.1	29.1
NH_3-N, mg/L	25.8	26.0	25.8	25.2
NO_3-N, mg/L	<0.1	0.1	0.1	0.1
NO_2-N, mg/L	<0.1	<0.1	<0.1	< 0.1
Turbidity, Jackson turbidity units	3.0	1.8	3.3	4.0
pH	7.65	7.70	7.70	7.70
Fe, mg/L	0.2	0.3	0.9	0.2
Mn, mg/L	0.10	0.13	0.03	0.03

TABLE 50. BACTERIOLOGICAL RESULTS ON OZONATION
(CSE Sample Set 1)

Item	Influent	Effluent	Log reduction
Total coliform per 100 mL	5.0×10^6	9.0×10^3	2.7
Fecal coliform per 100 mL	1.1×10^6	1.0×10^2	4.0

TABLE 51. ANALYTICAL DATA FOR THE OZONATION OF SAMPLE COLLECTED AT THE TAFT CENTER PILOT PLANT, CINCINNATI, OHIO
(CSE Sample Set 2)

Item	Ozonator	Diffusers 1	Diffusers 2	Diffusers 3
Power, W	700			
Pressure, kPa	58			
Gas dew point, °C	-78			
Gas temperature, °C	22			
Gas flow, L/min		40	21	21
O_3 conc. in, mg/L		8.2	8.2	8.2
O_3 conc. out, mg/L		0.4	0.2	0.4
O_3 utilized, %		95	98	95
SE flow, L/min		76	76	76
Applied dosage, mg/L		4.4	2.3	2.3
Effluent temperature, °C		17.0	17.0	17.0
O_3 residual, mg/L		1.1	0.8	0.4
Gas/liquid, L gas/L SE		0.54	0.28	0.28

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TABLE 52. CHEMICAL DATA FOR EFFLUENTS COLLECTED AT THE MEANDER CREEK WASTEWATER TREATMENT PLANT

	Sample Set 1		Sample Set 2	
	Raw	Final	Raw	Final
Date	6/15/78	6/15/78	10/5/78	10/5/78
Flow, m^3/s (MGD)		0.13 (3)		0.088 (2)
pH	8.1	8.15[a]	8.0	8.3[a]
NH_3-N, mg/L		2.1[a]		
PO_4-P, mg/L		7.2[a]		
NO_3-N, mg/L		5.7[a]		
NO_2-N, mg/L		0.007[a]		
COD, mg/L	980	30[a]	472	3.6[a]
BOD_5, mg/L	93	11[a]		
Suspended solids, mg/L		2.0[a]		
Ozone, dose, mg/L		5.4		6.0

[a] Undisinfected (control) effluent.

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sludge and clarification. The effluent is routinely chlorinated before discharge to the Hohokus Brook, which flows into Saddle River and ultimately the Passaic River. For purposes of determining the disinfection capabilities of ultraviolet light a portion of the effluent was passed through a uv-light irradiator. During warm temperature months, the plant operation was altered to include nitrification (ammonia removal) of the effluent. The treatment plant processes waste at flows of 0.24 m^3/s (5.5×10^6 gal/day).

Table 53 presents the available uv-irradiation treatment data for the two effluent collection periods at the uv-irradiation facility, as supplied by Dr. Karl Scheible, Hydro Qual, Inc.

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TABLE 53. PERFORMANCE DATA FOR THE ULTRAVIOLET DISINFECTION UNIT AT NORTHWEST BERGEN COUNTY WASTEWATER TREATMENT PLANT

	Sample Set	
	1	2[a]
Date	4/21/78	8/28/78
Sampling time	0930	1700
Total plant flow, m^3/s (MGD)	0.31 (7.0)	0.22 (5.0)
Banks of lamps	6	6
Lamps	386	360
Applied power, kWh	38.2	
Lamp temperature, °C	106	
Influent temperature, °C	13.4	
Wiper strokes per hour	6	
Exposure time, s	2.0	2.2
Relative intensity, $mW \cdot s/cm^2$	146,000	118,000
Applied voltage, v	460	
Influent total coliforms, MPN/100mL	2.4×10^5	4.9×10^5
Effluent total coliforms, MPN/100mL	80	79
Influent fecal coliforms, MPN/100mL	3.3×10^4	2.3×10^5
Effluent fecal coliforms, MPN/100mL	20	33
Influent COD-T,[b] mg/L	31	
Influent COD-F,[c] mg/L	20	
Effluent COD-T, mg/L	26	
Effluent COD-F, mg/L	26	
Influent color-F, platinum color units	30	
Effluent color-F, platinum color units	30	
Influent UV absorb.-T	0.134	
Influent UV absorb.-F	0.132	
Effluent UV absorb.-T	0.128	
Effluent UV absorb.-F	0.125	
Influent solids, mg/L	8.4	3.6
Effluent solids, mg/L	7.2	
Influent turbidity, Formazin turbidity units	2.5	2.8
Effluent turbidity, Formazin turbidity units	2.5	
Influent pH	7.2	6.8
Effluent pH	7.1	
Influent TKN-T, mg/L	20.2	
Influent TKN-F, mg/L	20.0	
Effluent TKN-T, mg/L	20.4	
Effluent TKN-F, mg/L	19.2	
Influent NH_3-N, mg/L	16.0	
Effluent NH_3-N, mg/L	16.8	

[a] Data limited because treatment plant program was not in an intensive sampling and analysis phase.
[b] T = total.
[c] F = filtered.

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